BIOGENESIS OF CHROMAFFIN GRANULES

Lynn Agnes Kilpatrick

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DECLARATION OF ORIGINALITY

I declare that the work presented in this thesis is my own and that this thesis was composed by me.

Department of Biochemistry,
University of Edinburgh Medical School,
Hugh Robson Building,
George Square,
Edinburgh EH8 9XD.

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ABSTRACT OF THESIS

Bovine adrenal medullary messenger RNA was isolated and translated in optimised reticulocyte lysate and wheatgerm cell-free translation systems. Reticulocyte lysate was found to be the superior translation system.

An attempt was made to characterize the primary polypeptide precursors to the major chromaffin granule secretory protein, chromogranin A, and to the two major chromaffin granule membrane proteins, dopamine β-hydroxylase and cytochrome b_{561}, using previously prepared and well-characterized antisera raised against these proteins. Two similar polypeptides of M_r 70,000 and pI of about 5.2 were immunoprecipitated from the translation products by the antiserum against chromogranin A. When reticulocyte lysate was supplemented with dog pancreas microsomes, one polypeptide of slightly lower M_r which was translocated into the lumen of the microsomal vesicles as determined by alkaline washing, was immunoprecipitated by this antiserum. Precursors to chromogranin A were subsequently identified from the polypeptide products when reticulocyte lysate was supplemented with adrenal medullary bound polysomes or rough microsomes. The effects of post-translational processing on chromogranin A were observed during the cellular synthesis of chromogranin A, during which chromogranin A becomes more heterogeneous with respect to pI and M_r. It is concluded that the smaller members of the chromogranin A family result from the action of intragranular proteolysis on chromogranin A during the maturation

and storage of the granules.

Two polypeptides of M_r 72,000 and 46,000, were immunoprecipitated from translation products by the antiserum raised against the soluble form of dopamine β-hydroxylase. The 46,000 dalton polypeptide is most likely a breakdown product of the 72,000 dalton polypeptide. When reticulocyte lysate was supplemented with dog pancreas microsomes, a polypeptide of M_r 67,000 daltons was immunoprecipitated by the antiserum to dopamine β-hydroxylase. This polypeptide was translocated into the lumen of the microsomal vesicles as determined by phase separation of the microsomes with Triton X-114. Thus, the soluble form of dopamine β-hydroxylase would appear to be synthesized as a precursor of 72,000 daltons which, on removal of its signal sequence, is reduced to a 67,000 dalton polypeptide.

Antisera were prepared against various chemically and enzymatically modified forms of cytochrome b_{561} in an attempt to immunoprecipitate the polypeptide precursor to this protein from translation products. However, all attempts to identify the precursor to this protein were unsuccessful.

An extensively-labelled small acidic translation product was tentatively identified as calmodulin.

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LIST OF ABBREVIATIONS

$A_{260\ (280,\ 235)}$, absorbance at 260nm (280nm, 235nm)(distance=1cm)

AMP,ADP,ATP, adenosine 5' mono-, di-, and triphosphate;

ATPase, adenosine triphosphatase;

$C_{12}E_8$, octaethylene glycol dodecyl ether;

CgA, chromogranin A;

Cyt. b_{561}, cytochrome b_{561}

DABITC, 4-NN-dimethylaminoazobenzene 4'-isothiocyanate;

DABTH, 4-NN-dimethylaminoazobenzene 4'-thiohydantoin

DBH, dopamine B-hydroxylase;

DEAE, diethylaminoethyl;

DNA, deoxyribonucleic acid;

dpm, disintegrations per minute;

EDTA, ethylenediamine tetraacetic acid;

EGTA, ethyleneglycol-bis-(B-amino ethyl ether) N'N'tetraacetic acid;

Fuc, fucose;

Gal, galactose;

Glc, glucose;

GTP, guanosine 5' triphosphate;

HEPES, N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid

man, mannose;

mRNA, messenger ribonucleic acid;

M_r, relative molecular mass;

NAcGalN, NGalAc, N-acetylgalactosamine;

NAcGlc, N-acetylglucosamine;

NANA, N-acetyl neuraminic acid;

NGNA, N-glycolyl neuraminic acid;

pI, isoelectric point;

PMSF, phenylmethylsulphonyl fluoride;

Poly A^+ RNA, polyadenylated ribonucleic acid;

PTH, phenylthiohydantoin;

TCA, trichloracetic acid;

TEMED, NNN'N'-tetramethyl-1,2-diaminoethane;

Tris, tris-(hydroxymethyl)aminomethane;

S. aureus, Staphylococcus aureus

SDS, sodium dodecyl sulphate;

SRP, signal recognition particle;

SUMMARY OF CONTENTS

CHAPTER 1

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

1.01.: INTRODUCTION

In recent years the processes involved in secretory organelle formation and protein secretion have been the subject of much research. The process of protein secretion has been proposed to occur as a series of six consecutive steps, namely secretory protein synthesis, segregation, intracellular transport, concentration, intracellular storage and discharge (Palade, 1975). Over the past ten years considerable progress has been made in understanding the mechanism of secretory protein synthesis and its segregation into the lumen of the endoplasmic reticulum (Walter et al., 1984). However, details of the biochemical mechanisms involved in intracellular transport and secretory organelle biogenesis have only recently begun to emerge (Palade, 1982) despite the fact that evidence for the assembly of secretory organelles in the Golgi apparatus was provided by electron microscopy in the 1950's (reviewed in Farquhar and Palade, 1981). Central to the problem of the biogenesis of the secretory organelle is the origin of its membrane. Are there proteins which specialize in the assembly of secretory organelle membranes? If so, the identification and biochemical characterization of such proteins would be crucial to our understanding of this process.

For some thirty years, the adrenal medulla has been the focus of much attention. It was proposed as a model system to study exocytosis after the observation that a granular fraction of adrenal medullary homogenate, containing the hormonal activity, could be

sedimented by centrifugation (Blaschko and Welch, 1953). Subsequently the ability to purify chromaffin granules on a large scale led to their becoming the best characterized secretory organelle (Winkler and Carmichael, 1982). The main function of the chromaffin granule is to accumulate, store and secrete adrenaline or noradrenaline. However, the catecholamines are co-stored and co-released with various chromaffin granule matrix proteins, including enkephalin-containing peptides and several soluble proteins of unknown function, known as chromogranins.

The secretory proteins of the chromaffin granule have been characterized by electrophoretic and immunological techniques (Fischer-Colbrie and Frischenschlager, 1985) as have the proteins of the granule membrane (Pryde and Phillips, 1985). As such the chromaffin granule offers a particularly good model system with which to study the biogenesis of secretory organelles, although at present there is very little detailed information on its assembly. Rather little has been achieved since early pulse-labelling experiments with whole glands were reviewed by Winkler (1977).

The object of this project was to study the initial events of the biosynthesis of the three most abundant and best-characterized chromaffin granule proteins; chromogranin A, the major secretory protein, dopamine β-hydroxylase, a major amphiphillic membrane protein which also exists in a soluble form, and cytochrome b_{561}, an integral transmembrane protein and the second most abundant chromaffin granule membrane protein. The precursors to these proteins were to be identified from the products of the *in vitro*

translation of adrenal medullary messenger RNA, by immunoprecipitation using previously-prepared and well-characterized antisera directed against these proteins. Subsequently, the in vitro processing of these proteins was to be investigated by employing dog pancreas microsomes in the translation reaction.

In this introduction, an attempt has been made to select and describe the major events known to occur in the "life-cycle" of a secretory organelle. This is followed by the introduction of the chromaffin granule and a summary of its composition.

1.02.01.:Protein Synthesis and Cotranslational Translocation across the Endoplasmic Reticulum

Early in vivo studies of secretory protein synthesis indicated that proteins destined for secretion were synthesized on polysomes bound to the endoplasmic reticulum (Siekevitz and Palade, 1960).

Detailed biochemical analysis of the processes involved in the translocation of secretory proteins across the membrane of the endoplasmic reticulum was facilitated by the development of in vitro cell free translation systems which, when programmed with dog pancreas microsomes, could reproduce the events which occurred in vivo (Blobel and Dobberstein, 1975b).

Proteins which are to be transferred from the cytoplasm across the membrane of the endoplasmic reticulum must first interact with it. After the observation that immunogobulin G light chains are

synthesized as slightly larger precursors in cell free translation systems in the absence of microsomes, Milstein suggested that such proteins are synthesized with an N-terminal peptide signal which directs them to the endoplasmic reticulum (Milstein et al., 1972). The "signal hypothesis" (Blobel and Dobberstein, 1975a) proposed that mRNA's encoding secretory proteins, encode also an amino terminal extension, or signal sequence, of 15-30 amino acids, which is subsequently removed by an endoprotease associated with the membrane of the endoplasmic reticulum. The majority of secretory proteins are synthesized in vitro as precursors with signal sequences, although ovalbumin, the major secretory protein of oviduct gland cells, is an exception, having an internal signal sequence (Palmiter et al., 1978). Comparison of the signal sequences from a large variety of proteins has shown that they vary both in length and amino acid sequence. However, all signal sequences contain a central region of hydrophobic residues with one or two basic amino acids at the amino terminus.

Translation in the wheatgerm cell-free system, of mRNA's encoding a secretory protein is initiated on free cytosolic polysomes, but as the signal sequence emerges from the large ribosomal subunit (when the polypeptide is between 60-80 amino acids long) further elongation is halted (Walter and Blobel, 1981) by the high affinity binding of a signal recognition particle (SRP) (Walter et al., 1981). However, recently it has been suggested that elongation arrest may be a peculiarity of wheatgerm extract (Walter, 1985). Translation of messenger RNA's encoding secretory proteins in reticulocyte lysate or HeLa cell lysate is not halted by the binding

of SRP (Walter, 1985). Whether or not the SRP-mediated elongation arrest is a genuine phenomenon *in vivo* remains to be determined. The mechanism by which the SRP interacts with the ribosome and signal sequence is unknown. The secondary or tertiary structure of the signal sequence must be involved with the recognition and/or interaction of the SRP because perturbation of the signal sequence, by the incorporation of amino acid analogues, renders it unrecognizable (Hortin and Boime, 1980a).

The SRP is an 11S cytoplasmic ribonucleoprotein composed of six distinct polypeptide chains of 72, 68, 54, 19, 14, and 9 kilodaltons (Walter and Blobel, 1980) and a 7S RNA molecule (Walter and Blobel, 1982). Electron micrographs show that it is a rod-like particle of 24nmx5nm (Andrews *et al.*, 1985). The 7S RNA is about 300 nucleotides long containing a central "S segment" of 155 nucleotides with a unique sequence flanked by sequences of 100 and 50 nucleotides at the 5' and 3' ends respectively. The Alu family is an abundant family of repetitive DNA sequences of about 300 base pairs which are highly interspersed throughout the genome. They have been found in the neighbourhood of all structural genes isolated to date. The sequences flanking the "S segment" of the 7S RNA molecule exhibit homology with the Alu family (Ullu *et al.*, 1982). The 7S RNA apparently acts as a structural backbone in the assembled SRP although it may have other, as yet unknown, functions.

Limited nucleolytic digestion splits the SRP into two domains, one containing the 72, 68, 54, and 19 kilodalton polypeptides, and the other containing the 14 and 9 kilodalton polypeptides. *In vitro*

reconstitution experiments with the SRP in wheatgerm extract have shown that, in the absence of the 14 and 9 kilodalton polypeptides, the SRP fails to arrest elongation although it still mediates the translocation of a secretory protein across the membrane of the endoplasmic reticulum (Siegel and Walter, 1985). This implies that elongation is not a prerequisite for protein translocation. The domain of the SRP containing the 9 and 14 kilodalton polypeptides may function to specifically arrest elongation whereas the domain containing the 72, 68, 54, and 19 kilodalton polypeptides may be involved in assisting translocation. It has been suggested (Siegel and Walter, 1985) that elongation arrest has arisen as an evolutionary refinement and may serve to increase the efficiency of translocation and prevent the complete synthesis of secretory protein precursors in the cytoplasm. It may also provide the cell with a switch for controlling the synthesis of secretory proteins. These suggestions may of course be invalid if elongation arrest is peculiar to wheatgerm extract as discussed above.

Elongation arrest is released when the ribosome-bound SRP interacts with the SRP receptor (Gilmore et al., 1982) also known as the docking protein (Meyer et al.,1982), located on the membrane of the endoplasmic reticulum. The docking protein is an integral membrane protein of 72 kilodaltons of which 60 kilodaltons forms a cytoplasmic domain (Meyer and Dobberstein, 1980a; 1980b). The cytoplasmic domain can be proteolytically removed from microsomes, rendering them incapable of translocation. Activity can however be restored by recombining the fractions. The SRP and the docking protein both appear to require free sulphydryl groups for their

activity (Jackson et al., 1980).

The SRP's affinity for the ribosome is reduced by its transient interaction with the docking protein (Gilmore and Blobel, 1983). Displacement of the SRP is accompanied by the formation of a ribosome-microsome junction, permitting cotranslational translocation of the secretory protein. The docking protein is not thought to be directly involved in the formation of this junction since it does not show any measurable affinity for ribosomes, but is required for the translocation process (Siegel and Walter, 1985). The docking protein may function to direct the SRP-bound ribosome to the microsomal membrane. It may also be involved with the initiation of the translocation process directly, or it may organize other components required for this process. After the formation of the ribosome-membrane junction, probably by the interaction of the ribosome with other integral endoplasmic reticulum membrane proteins, the SRP and the docking protein are free to repeat the process (see Fig.1.01.). Two integral membrane glycoproteins of the endoplasmic reticulum, ribophorins I and II, bind ionically to ribosomes (Kreibich et al., 1978). However, there is no evidence for their involvement in the formation of the ribosome membrane junction or protein translocation.

The SRP and the docking protein are intimately involved with the recognition and direction of proteins with endoplasmic reticulum-directed signal sequences to the correct subcellular location. As such the components involved with cotranslational protein translocation have been highly conserved throughout

Fig. 1.01. SRP CYCLE AND RIBOSOME CYCLE

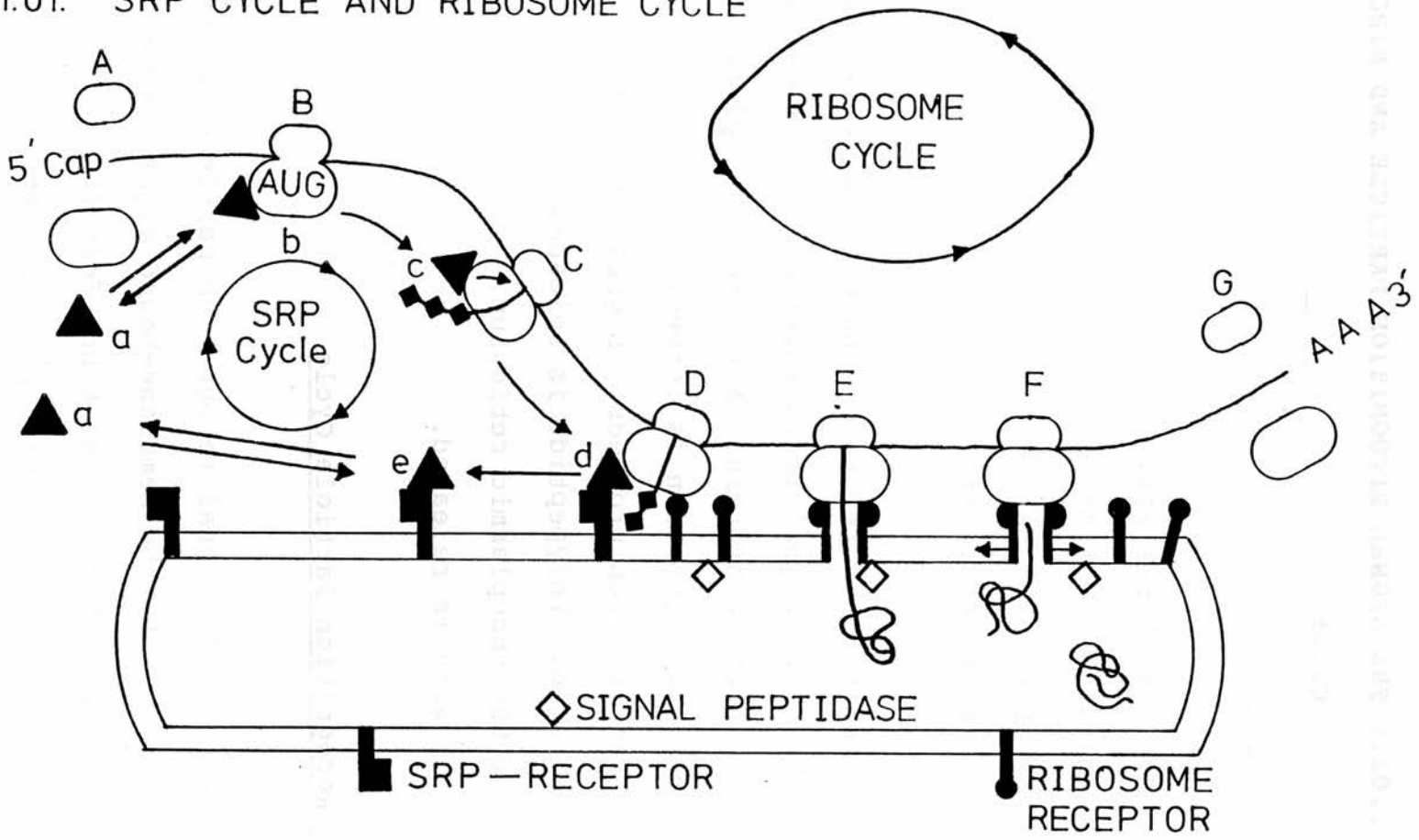


FIG.: 1.01.: THE SIGNAL RECOGNITION PARTICLE AND RIBOSOME CYCLE (from Walter et al., 1983)

Ribosome Cycle

A,B. Ribosome binds mRNA encoding a secretory protein; initation of translation; elongation of polypeptide

C. Signal Recognition Particle interacts with signal sequence

D. Ribosome is directed to endoplasmic reticulum where the signal recognition particle docks with the docking protein. A ribosome/endoplasmic reticulum junction is formed.

E.F. Translocation proceeds. Signal sequence is cleaved. Polypeptide is released into the lumen of the endoplasmic reticulum.

G. Ribosome is released.

Signal Recognition Particle Cycle

The soluble signal recognition particle (a) exists in equilibrium with the membrane-bound signal recognition particle (e) and the ribosome-bound form (b).

(c) The signal recognition particle interacts with the signal sequence of the nascent polypeptide.

(d) The signal recognition particle interacts with the docking protein on the endoplasmic reticulum.

evolution. This is demonstrated by the ability to synthesize and correctly process proteins using heterologous components *in vitro*. For example plant (wheatgerm) ribosomes in conjunction with mammalian (canine) microsomes can synthesize and correctly process prokaryotic secretory proteins.

The mechanism which permits the transfer of a protein across the hydrophobic barrier presented by the membrane is as yet unknown (reviewed in Sabatini *et al.*, 1982). Translocation could be assisted by the formation of a water-filled proteinaceous channel (Blobel and Dobberstein, 1975a), the formation of which may be initiated by a conformational change in an integral protein such as the docking protein or the ribophorins. Energy for translocation may be provided by the binding of ribosomes to receptors or by protein synthesis (Sabatini *et al.*, 1982). Alternatively it has been suggested, on thermodynamic grounds, that protein translocation could occur without the involvement of membrane proteins (Engelman and Steitz, 1981). Translocation across the endoplasmic reticulum does not appear to require an electrical potential nor an ion gradient since uncouplers and ionophores have no effect on protein translocation *in vitro* (Sabatini *et al.*, 1982).

Cotranslational translocation is mediated by a variety of enzyme activities which may serve to make the process irreversible. Signal sequences are removed in most cases by a signal peptidase. This is an integral membrane protein on the luminal surface of the membrane of the endoplasmic reticulum (Kreibich *et al.*, 1980). Characterization of this enzyme has been unsuccessful due to

difficulties in assaying it and inhibiting its activity (Jackson and Blobel, 1980). The structural features of signal sequences which are recognized by the signal peptidase appear to have been conserved throughout evolution. The cleaved signal peptides are probably rapidly hydrolyzed since all attempts to detect them have been unsuccessful (Jackson and Blobel, 1977).

Other events that occur cotranslationally are the transfer of high-mannose core oligosaccharides to specific asparagine residues, initiating N-linked glycosylation (Section 1.03.04.), and the formation of disulphide bonds.

On termination of protein synthesis, the secretory protein is released into the lumen of the endoplasmic reticulum, the ribosome is released from the membrane and the membrane of the endoplasmic reticulum once more becomes impermeable to proteins (Fig.1.01.).

Secretory proteins are not the only proteins to be cotranslationally translocated across the endoplasmic reticulum. Lysosomal proteins (Erickson et al., 1983) and certain classes of integral membrane proteins (Lingappa et al., 1978) also use this mode of translocation. Proteins destined for secretion or lysosomes are translocated wholly across the membrane of the endoplasmic reticulum into the lumen. However, proteins destined to remain integrated within a membrane somehow interrupt translocation prior to the completion of the polypeptide chain. The orientation of an integral transmembrane protein in the endoplasmic reticulum is identical with that of the final destination.

1.02.02.: Halt Transfer Sequences

Much information on the biogenesis of membrane proteins has been obtained from the study of a few well-characterized membrane proteins including glycophorin A, histocompatibility antigens, immunoglobulin M heavy chains, vesicular stomatitis virus G-protein and various other viral glycoproteins. The membrane domains of such proteins have been examined in order to gain some insight into potential "halt transfer signals". Such domains have been found to contain a hydrophobic segment of 20-32 amino acids, followed by a group of positively charged amino acids, thought to be exposed to the cytoplasm. However a stretch of hydrophobic amino acids is not in itself sufficient to halt translocation, since some membrane proteins have long stretches of hydrophobic amino acids which are translocated across the endoplasmic reticulum.

Valuable information on "halt transfer sequences" and signal sequences has been obtained by the creation of hybrid proteins using recombinant DNA technology (Bassford et al., 1979). It is possible to convert a secretory protein, such as rat growth hormone, into a transmembrane protein by fusing the 3' terminus of the gene coding for the growth hormone with the 5' terminus of the gene sequences encoding the membrane spanning and cytoplasmic domains of vesicular stomatitis virus G-protein (Guan and Rose, 1984). The resulting hybrid protein, which was recognized by antibodies to both of the parent proteins, became anchored in microsomal membranes, with the growth hormone moiety exposed to the lumen. The hybrid protein was transported to the Golgi but not to the cell surface. However,

when the gene encoding a non-secretory protein, globin, was fused with that of a secretory protein, β-lactamase, the hybrid protein was secreted. The insertion of the sequence encoding a transmembrane region of an integral membrane protein, immunoglobulin M heavy chain, between the coding regions of globin and lactamase did, however, result in the new hybrid protein behaving as a transmembrane protein (Yost etal., 1983). This suggests that signal sequences and stop transfer sequences are distinct and although both sequences are necessarily hydrophobic, some other property must allow hydrophobic signal sequences to be distinguished from hydrophobic stop transfer sequences. These experiments do not support the idea that translocation across the endoplasmic reticulum would be able to occur spontaneously solely on the basis of hydrophobic interaction between the membrane and hydrophobic regions of the nascent polypeptide (Engelman and Steitz, 1981).

Amino acid sequences, such as signal sequences and halt transfer sequences, which dictate the cellular location of a protein, have been named "topogenic sequences" (Blobel, 1980). The control that a topogenic sequence exerts over a protein is well demonstrated in vivo by the β-lymphocyte which produces plasma membrane associated immunoglobulin M and secretory immunoglobulin M (Early et al., 1980). One gene produces, by different splicing events, two different mRNA molecules which encode proteins differing only at their carboxyl terminus. The membrane bound form of immunoglobulin M has a hydrophobic sequence of 26 amino acids which presumably anchors it to the membrane. This hydrophobic sequence is flanked by 12 negatively-charged amino acids at its amino terminal side, and by a tripeptide containing two lysine residues on its carboxyl terminus

(cytoplasmic) side. Secretory immunoglobulin M lacks this carboxyl terminus and instead has developed a carboxyl terminus with residues optimal for interaction with an aqueous environment.

1.02.03.: Post-Translational Translocation

An alternative mode of translocation of proteins across membranes does exist and will be mentioned briefly.

Cytoplasmically synthesized proteins destined for peroxisomes, mitochondria or chloroplasts are transferred across the membrane(s) of these organelles independently of translation. The best studied system is the import of proteins into mitochondria (Hay et al., 1984). Such proteins are synthesized as larger precursors which are proteolytically processed after translocation. The precursors have amino terminal extensions which are structurally and functionally different from signal sequences of secretory proteins in that they are larger, they are not hydrophobic and they are probably not directly responsible for directing the polypeptide to the appropriate organelle. Instead these extensions may in some cases affect the configuration of the protein, making it recognisable to surface receptors on the organelle. This is comparable to the membrane trigger hypothesis, proposed as an alternative to the signal hypothesis for secretory proteins (Wickner, 1979). Subsequent cleavage of the extension may alter the conformation of the polypeptide which, in turn, may serve to retain the polypeptide within the organelle. Translocation of mitochondrial proteins is dependent on the mitochondrial membrane potential, and appears to

involve receptors on the cytoplasmic face of the mitochondrion.

1.03.: INTRACELLULAR TRANSPORT

Intracellular transport requires energy in the form of adenosine triphosphate since, in its absence, newly synthesized secretory proteins remain in the endoplasmic reticulum (Jamieson and Palade, 1968). Palade (1975) suggested that transport between the endoplasmic reticulum and the Golgi apparatus was mediated by vesicles which shuttle between the transitional elements of the rough endoplasmic reticulum and the cis-most cisternae of the Golgi apparatus.

Much of the information now available on the transport of proteins to and their passage through the Golgi apparatus has been obtained by studying the cellular transport of viral membrane proteins following viral infection. The virus in an infected cell takes over the protein synthesizing machinery of the cell for the synthesis of its own viral proteins. The proteins encoded by vesicular stomatitis virus and Semliki forest virus are well-characterized and consequently, cells infected with either of these viruses provide good model systems with which to work.

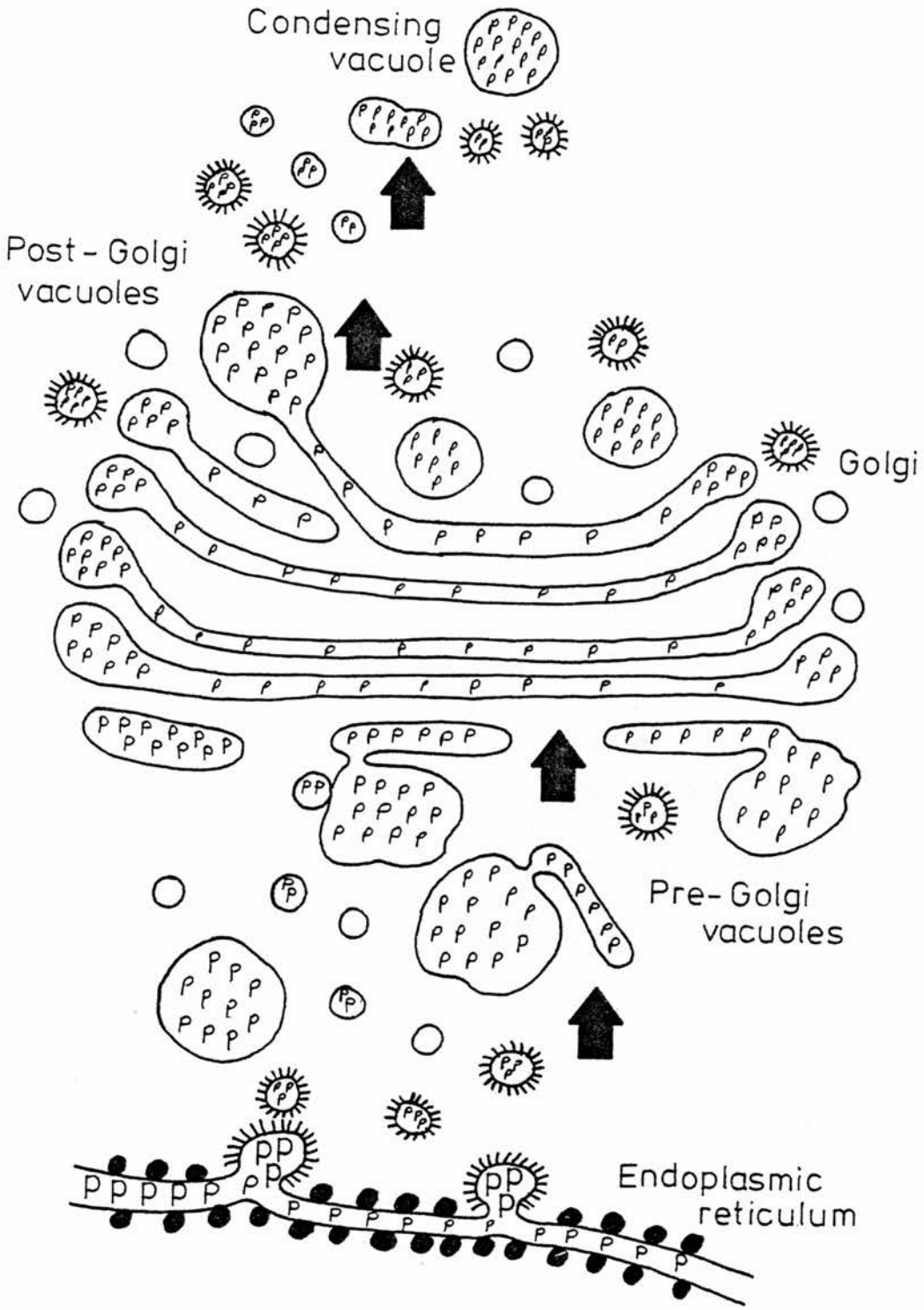


Fig. 1.02. TRANSPORT OF A PROTEIN (P) THROUGH THE GOLGI (from Saraste and Kuismanen, 1984).

1.03.01.:Transport of Proteins from the Endoplasmic Reticulum to the Golgi Apparatus

The ability to reversibly block intracellular protein transport has enabled the identification of components involved with protein transport between the endoplasmic reticulum and the Golgi apparatus by immunocytochemistry coupled with electron microscopy. The intracellular protein transportation of two viral membrane glycoproteins was synchronized using baby hamster kidney fibroblasts infected with a temperature-sensitive mutant of Semliki forest virus. At the restrictive temperature of 39^{o}C, viral proteins were trapped in the endoplasmic reticulum. However, intracellular movement of the proteins was resumed by incubating the cells at 28^{o}C (Saraste and Headman, 1983). Coated and smooth 100nm vesicles appeared to be involved in the transport of proteins from the endoplasmic reticulum to the Golgi apparatus. However, when incubated at 15^{o}C, viral proteins were transported out of the endoplasmic reticulum, but were then trapped in pre-Golgi vacuoles (Fig. 1.02.; Saraste and Kuismanen, 1984). Pre-Golgi vacuoles may be created by the fusion of small primary vesicles. Alternatively, they may be genuine organelles to which proteins are transported by the small primary vesicles. It is likely that the pre-Golgi vacuoles fuse together to form the extreme cis cisternae of the Golgi.(Saraste and Kuismanen, 1984). More details of intracellular transport between the endoplasmic reticulum and the Golgi apparatus, and the nature of the vesicles involved, will probably be obtained from such systems in the future, using reversible blocks to synchronize the transport of proteins.

Several lines of investigation have indicated that transport from the endoplasmic reticulum to the Golgi apparatus is not a passive process. In spite of being transported via the same Golgi route (Strous et al., 1983) secretory proteins are transported at different rates from the endoplasmic reticulum to the Golgi (Fries et al., 1984). This phenomenon may arise as the result of "signals" on the (glyco)proteins determining the affinity with which they bind receptors involved in mediating their transport (Fitting and Kabat, 1982; Lodish et al., 1983).

1.03.02. :Intracellular Transport through the Golgi

The Golgi apparatus is centrally positioned in the pathway of intracellular protein transport. Proteins exported from the endoplasmic reticulum enter the Golgi at the cis face and exit from the trans face (reviewed in Palade, 1975; Farquhar and Palade, 1981). Transport of proteins through the Golgi is often accompanied by a series of precise structural modifications such as fatty acylation, sulphation, phosphorylation, O-linked glycosylation and the modification of N-linked core oligosaccharides. As they are transported through the Golgi, the proteins are sorted and directed to their correct destination (Tartakoff, 1980). The elucidation of the mechanism and precise route of transport of proteins through the Golgi will aid the understanding of the sorting process.

1.03.03.: The Organization of the Golgi.

The central feature of the Golgi is a stack of between 4 and 15 smooth cisternae which usually have flattened platelike centres with dilated rims. Coated vesicles are intimately associated with the Golgi stacks whereas organelles such as mitochondria, ribosomes and rough and smooth endoplasmic reticulum, although seen in the vicinity of the Golgi, are excluded from the region of the Golgi stack. Golgi cisternae are heterogeneous and the Golgi stack is asymmetrical as demonstrated by morphological and cytochemical studies. The cis Golgi cisternae are closest to the endoplasmic reticulum and are often slightly curved towards it. The membranes of the cis cisternae appear thinner and are preferentially stained by osmium tetroxide. Also of interest is the fact that cis Golgi cisternae often contain enzymes characteristic of the endoplasmic reticulum, such as glucose-6-phosphatase, a marker enzyme of the endoplasmic reticulum in cells active in gluconeogenesis i.e. liver and kidney. The trans Golgi cisternae are furthest from the endoplasmic reticulum and often curve away from it. Acid phosphatase and thiamine pyrophosphatase activities are restricted to one or two of the trans-most cisternae. Lectin binding also demonstrates some differences between cis and trans Golgi cisternae since particular protein-linked oligosaccharides will only occur in specific Golgi regions after having been processed by the appropriate glycosylases.

1.03.04.: Processing of N-glycosidically Linked Oligosaccharides to Monitor Transport of a Protein through the Golgi

Conjugation of carbohydrate to proteins can be mediated either by N-linkage via an asparagine residue or by O-linkage via a serine or threonine residue. The biosynthesis of N-linked oligosaccharides, despite its complexity, is better understood than the biosynthesis of O-linked oligosaccharides.

N-linked glycosylation is initiated by a cotranslational event involving the transfer of $Glu_3Man_9NGlcAc_2$ from the carrier lipid moiety dolichol phosphate, to specific asparagine residues of nascent polypeptides (Hubbard and Ivatt, 1981). Glucose residues are then removed by glucosidases I and II in the endoplasmic reticulum to generate $Man_9N\text{-}GlcAc_2$. The high mannose core may then be processed either to smaller high mannose oligosaccharides (Bischoff and Kornfeld, 1983) or, more commonly, to complex oligosaccharides by mannosidase I, located in an early region of the Golgi, which converts the oligosaccharide to $man_5N\text{-}GlcAc_2$. Further modifications to N-linked oligosaccharides are carried out by a series of enzymes including N-acetylglucosaminyl transferases I, II, III and IV, α-mannosidase II, fucosyl transferase, and galactosyl and sialyl transferases (Hubbard and Ivatt, 1981). These modifications make the glycoprotein resistant to endoglycosidase H and sensitivity or resistance to this enzyme can therefore be used to monitor the extent of transport of the protein through the Golgi.

In contrast O-linked glycosylation is a post-translational process, initiated in an early region of the Golgi (Roth, 1984), by the addition of N-acetylgalactosamine. Subsequent addition of fucose, N-acetylglucosamine, galactose and sialic acid residues occurs later in the Golgi and is apparently concomitant with the later processing events of N-linked oligosaccharides.

1.03.05.: The Golgi Comprises Three Functionally Distinct Compartments

The first indication that the Golgi stack can be divided into three functionally distinct compartments (cis, medial and trans) as opposed to two (cis and trans), was from a combination of cytochemical, immunocytochemical and biochemical studies on Semliki forest virus-infected baby hamster kidney cells, treated with monensin (Griffiths et al., 1983). Monensin, a sodium ionophore, blocks intracellular transport without affecting protein synthesis, and causes an accumulation of immature proteins at the mid (medial) Golgi cisternae, which are cytochemically and immunocytochemically distinct from early (cis) and late (trans) Golgi cisternae. Monensin therefore blocks intracellular transport from the medial Golgi to the trans Golgi. The Golgi cisternae harbouring the accumulated viral glycoprotein intermediates were isolated, utilizing their altered physical properties, and the modifications that the protein had undergone en route to that intracellular location were investigated (Quinn et al., 1983). This study revealed that fatty acylation occurred either in the cis or medial cisternae, and prior to trimming and construction of complex N-linked oligosaccharides.

However, the intracellular location of the enzyme activities involved in the trimming and construction of N-linked oligosaccharides could not be resolved in this study.

More recently, Rothman et al., (1984b) have located various Golgi associated enzyme activities and given an indication as to how proteins are intracellularly transported between Golgi compartments.

1.03.06.: Intercompartmental Transfer of Proteins Through the Golgi Stack is a Vectorial Process

The mechanism of transfer of proteins across the Golgi stack has been investigated by studying the transfer of the vesicular stomatitis G-protein through the Golgi of Chinese hamster ovary cells which lack N-acetylglucosaminyl transferase (Rothman et al., 1984a; reviewed by Rothman, 1985). The modifications that this viral glycoprotein undergoes as it passes through the Golgi are well documented (Dunphy et al., 1981). It is fatty-acylated in the cis-Golgi compartment and then its N-linked precursor oligosaccharide is trimmed by mannosidases in the medial Golgi. Normally the terminal sugars N-acetylglucosamine, galactose and sialic acid are then added by their respective transferases. However when mutant Chinese hamster ovary cells, deficient in N-acetylglucosaminyl transferase, are infected with vesicular stomatitis virus and then fused with cells capable of adding N-acetylglucosamine, the glycoprotein was transported to the Golgi region where galactose and sialic acid could be incorporated (Rothman et al., 1984b). This

process occurred at the same rate in the fused mutant cells as it did in a single wild type cell. This suggests that the transfer of proteins between Golgi compartments occurs by a dissociative process with the budding of transport (coated) vesicles from cisternae followed by their fusion with a more distal Golgi stack (reviewed in Dunphy and Rothman, 1985; Rothman, 1985). If the fusion was performed at a later stage, after the transfer of the glycoprotein to a Golgi region distal to that of the deficient activity, the glycoprotein could not be transferred to the Golgi stack with the active enzyme, implying that Golgi transport is a vectorial process. The experiments support the idea of there being three biochemically distinct Golgi compartments:- the cis Golgi involved with fatty acylation, the medial Golgi housing mannosidases I and II, fucosyl and N-acetylglucosaminyl transferases, and the trans Golgi, housing galactosyl and sialyl transferases. Further divisions within these compartments may exist and mutant cells, deficient in the appropriate enzyme activities, will enable their identification. However, since some cells contain as few as 4 Golgi stacks, no more than this number of distinct compartments would be expected to exist.

1.03.07.: The Problem of Sorting

The mechanism by which the Golgi apparatus sorts and directs proteins to their correct destination is unknown. Cells specialising in secretion must segregate lysosomal enzymes from secretory proteins and must also direct membrane proteins to their correct destinations. Mannose-6-phosphate residues are uniquely located on lysosomal enzymes and accumulating evidence suggests that mannose-6-phosphate receptors, which are found throughout the Golgi, are involved in the segregation of lysosomal proteins from secretory proteins (Geuze et al., 1984). During their transport through the Golgi apparatus by a continuous process of vesicle budding and fusion, secretory proteins must be provided with a "membrane container". How is this membrane domain created and furthermore, how is it maintained? It is likely that there are proteins which specialize in this process and therefore may be expected to be components of every secretory granule membrane.

Another process in which the early Golgi is involved is the sorting of endoplasmic reticulum membrane proteins and their return to this compartment (Rothman, 1982). Rothman suggests that the early Golgi cisternae function in a way analogous to a distillation tower, repeatedly refining secretory and secretory granule membrane proteins until only the cleanest fractions arrive at the trans Golgi cisternae.

1.04.:CONCENTRATION

Concentration is not an obligatory step in secretory organelle biogenesis but occurs in the majority of secretory cells. It is descriptive of all the post-Golgi processes leading to the formation of the mature secretory granules and may include not only the concentration of secretory proteins, but also the accumulation and concentration of small molecules, for example Ca^{2+}, ATP, and catecholamines, within prosecretory granules.

Concentration of secretory proteins occurs in the dilated rims of the trans-most Golgi cisternae, giving rise to post-Golgi vacuoles (Saraste and Kuismanen, 1984, and see Fig. 1.02.). This process is not dependent on a continuous supply of energy as would be expected if ion-pumping mechanisms were involved. Instead, concentration may be a result of complex formation between secretory proteins, which are usually cationic, and polyanions such as mucopolysaccharides. Calcium ions may also play a role in these aggregation processes(Palade, 1975; Farquhar and Palade, 1981). Clathrin coated trans-Golgi membrane regions have been identified (Orci et al., 1984) from which coated vesicles bud. Subsequent uncoating and fusion of these vesicles gives rise to condensing vacuoles (Fig. 1.02.). Secretory proteins are further concentrated within the condensing vacuole by shuttling coated vesicles transporting secretory proteins from the trans-Golgi stack. In many cases secretory proteins are synthesized as larger proproteins e.g. proinsulin, proenkephalin. Maturation of such proteins, by the action of specific proteases, appears to occur during the formation and maturation of the secretory

granule (Steiner et al., 1984).

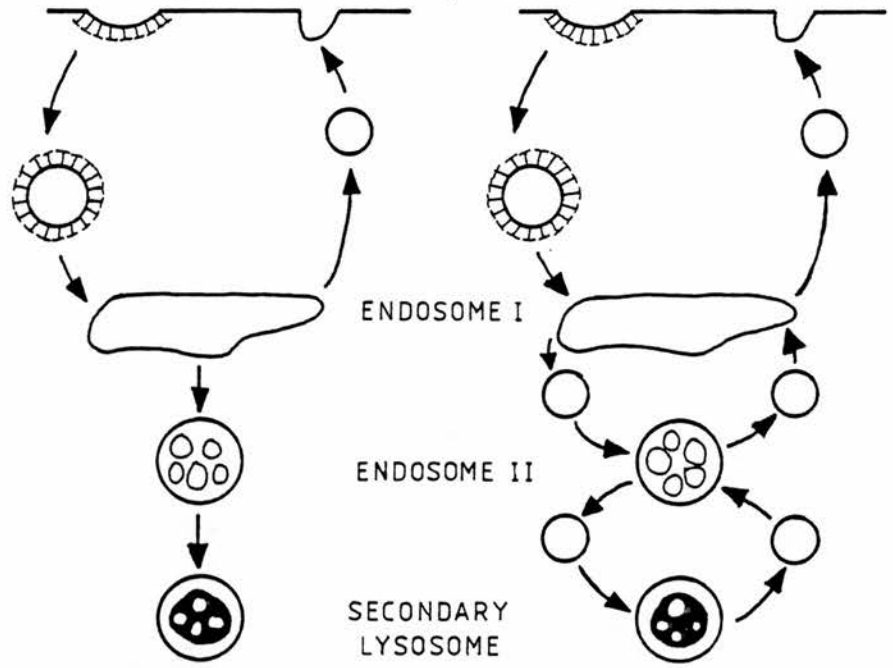
1.05.01.:Intracellular Storage and the Secretory Cycle

Secretory proteins are stored within secretory granules, often as a mixture and often along with other smaller molecules. Although the secretory granule membrane is Golgi-derived, it is of a different, generally simpler, protein composition than Golgi, endoplasmic reticulum or plasma membranes. The membranes of secretory granules, Golgi and plasmalemma are by necessity impermeable, due to their high content of cholesterol, sphingomyelin, and saturated fatty acids. In contrast, the highly permeable membrane of the endoplasmic reticulum contains more phosphatidylcholine and less sphingomyelin (Farquhar and Palade, 1981).

Secretory granules are often stored in an apical region of the cell, between the trans Golgi and the cell membrane, suggestive of a translocation mechanism.

Appropriate stimulation of the cell, resulting in an increase in the intracellular calcium concentration, leads to the translocation of the secretory granule to the plasma membrane followed by discharge of the granule contents from the cell by exocytosis which requires the fusion of the secretory granule and plasma membranes. The nature of the "signalling" mechanism(s) triggered by an increase in the intracellular calcium concentration, and leading to exocytosis, is unknown but protein phosphorylation is

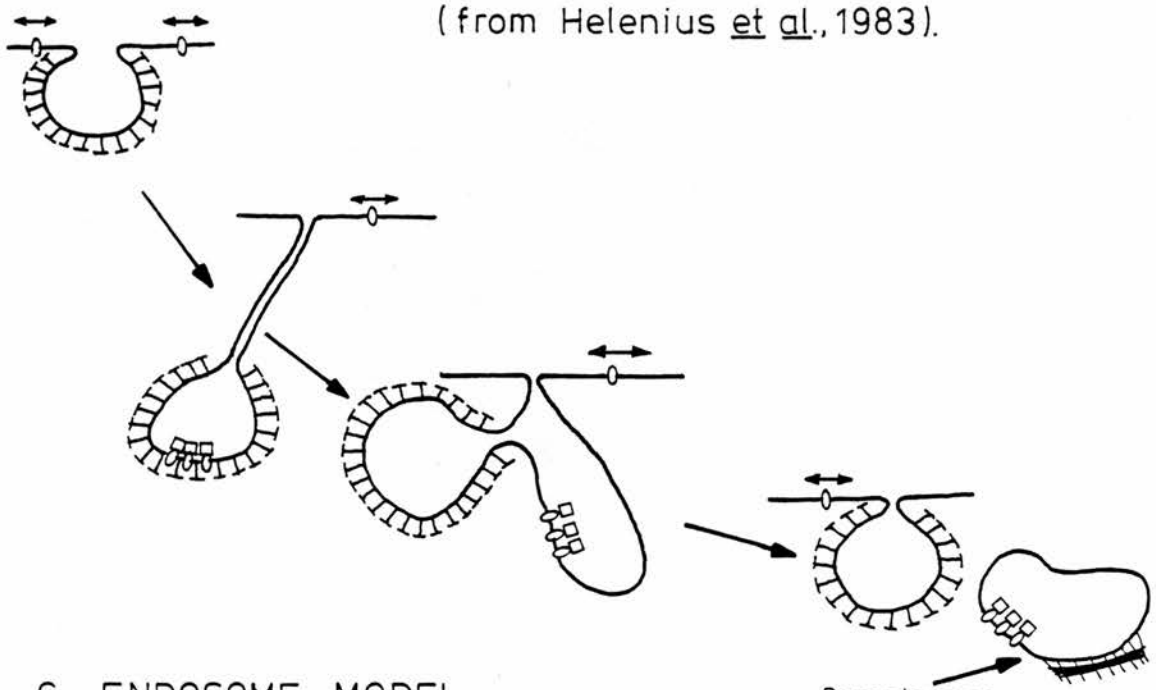
Fig. 1.03. POSSIBLE MODELS FOR THE INTERNALIZATION OF A COATED PIT.



A. MATURATION MODEL.

B. VESICLE SHUTTLE MODEL.

MATURATION AND VESICLE SHUTTLE MODELS (from Helenius et al., 1983).



C. ENDOSOME MODEL. (from Pastan and Willingham, 1983)

strongly implicated (see Section 1.12.).

Exocytosis is invariably followed by endocytosis, the retrieval of the granule membrane (reviewed in Farquhar, 1983). Much information about endocytosis has been obtained from studies of receptor mediated endocytosis of low density lipoprotein and of peptide hormones, and of macrophage membrane recycling. The endocytosis of material from the cell surface is initiated by the recruitment of the coating protein, clathrin (Pearse, 1976) to the plasma membrane, a process which is Ca^{2+} and calmodulin dependent (Salisbury et al., 1981), possibly through the action of a Ca^{2+}/calmodulin-dependent protein kinase on certain proteins which may subsequently be recognized by clathrin (Moskovitz et al., 1983). This nucleation event is accompanied by the further addition of clathrin (Unanue et al., 1981) and the growth of a "coated-pit". The next event is a point of some controversy. The most favoured mechanism is that the coated pit pinches off to form a coated vesicle which rapidly loses its coat and fuses with an endosome (Helenius et al., 1983, see Fig.1.03.A.). The alternative mechanism suggests that coated pits remain permanently associated with the cell surface and that "receptosomes" bud directly from a region adjacent to the coated pit (Pastan and Willingham, 1983, see Fig.1.03.B.). Whatever the mechanism for the internalization of the pit, the retrieved membranes are probably incorporated into endosomes (receptosomes) for an "acid wash". Endosomes are prelysosomal compartments, lacking lysosomal enzymes, but with an acidic interior. The washed, reinternalized secretory granule membranes are then returned to a region of the

Golgi, possibly the trans Golgi (Farquhar, 1983), for repackaging of secretory proteins and granule maturation.

1.05.02.: Multiple Transport Roles of Coated Vesicles

Coated vesicles have been likened to a circulatory system for the cell (Brown et al., 1983). They are implicated in transport from the endoplasmic reticulum to the Golgi (Section 1.03.02.), transport through the Golgi stacks (Section 1.03.06.) in the condensation process (Section 1.04.) and in membrane recovery and recycling (Section 1.05.01.). Presumably there are different populations of coated vesicles with different transport functions.

The major component of coated vesicles is the coating protein, clathrin (180 kilodaltons) and two clathrin light chains (34 and 32 kilodaltons) which are always found in association with coated vesicles. The assembly of the clathrin trimers (consisting of trimers of clathrin in association with three clathrin light chains) to form polyhedral cages has been studied in vitro (Crowther and Pearse, 1981) and occurs spontaneously at pH6-6.5 in the presence of Mg^{2+} or Ca^{2+}.

Clathrin-coated vesicles contain an ATP-dependent proton pump which serves to acidify the vesicle interior (Forgac et al., 1983). Acidification may be required for the fusion of the coated vesicle with its target membrane, as is the case for the fusion of, for example, the membrane of Semliki forest virus with lysosomal

membranes to release the viral genome into the host cell cytoplasm (Helenius et al., 1980). Shortly after budding, the clathrin coat is removed from the coated vesicle by an uncoating ATP'ase (Schlossman et al., 1984).

1.06.:The Constitutive Pathway for Secretion

The secretion of proteins from cells such as plasma cells, fibroblasts and macrophages is not subject to short term regulation by extracellular calcium and is insensitive to cAMP (Tartakoff et al., 1978). Such cells use a constitutive pathway for secretion whereby newly synthesized secretory proteins are rapidly transported in Golgi derived vesicles to the plasma membrane and are released extracellularly by exocytosis. It has been suggested that this pathway could be the one used by all cells for the insertion of plasma membrane proteins (Gumbiner and Kelly, 1982) and as such would be required for cell viability. The alternative secretory pathway (1.05.01.), regulated by extracellular calcium and sensitive to cAMP, is only present in cells which specialize in secretion such as endocrine, exocrine and neuronal cells.

1.07.01: THE ADRENAL MEDULLA

The adrenal gland is a comparatively small organ situated above the kidney. It is composed of two distinct populations of cells, those of the medulla which are involved primarily with the biosynthesis, storage and secretion of catecholamines, and those of the cortex which synthesize steroid hormones. The bovine adrenal medulla is easily dissected from the surrounding cortex by slicing the gland horizontally and scraping out the pinkish medulla. This procedure inevitably results in medullary preparations being contaminated to some extent with cortical material, a fact which must always be borne in mind.

Embryologically, the adrenal medulla originates from the neural crest, as do the cells of the sympathetic nervous system. It is impossible to prepare an homogenous population of sympathetic neurones owing to the long processes with which they innervate, sometimes distant, organs. However, the cells of the adrenal medulla are cuboidal and as such, the adrenal medulla is a source of an easily prepared, homogeneous population of "model" nerve cells.

The adrenal medulla is extremely well vascularized and innervated, and is composed of groups and columns of chromaffin cells, so called due to their reaction with chromium salts (which stain the catecholamine-storing granules brown). There are two types of chromaffin cell, each storing and releasing either adrenaline or

TABLE 1.01.

COMPONENTS OF THE CHROMAFFIN GRANULE MATRIX

(from Njus et al., 1981, and Phillips and Apps, 1979)

Catecholamines	600mM
Nucleotides	220mM
ATP	160mM
Ca^{2+}	20-30mM
Mg^{2+}	6mM
Ascorbate	22mM
Soluble protein	200mg/ml
pH	5.5

noradrenaline. The proportion of these cells varies between species. Splanchnic nerve stimulation in response to stress releases acetylcholine, which in turn depolarises the chromaffin cells causing an increase in the intracellular calcium concentration. This increased calcium concentration mediates the release of catecholamines by exocytosis. Adrenaline acts to stimulate the rate of liver and skeletal muscle glycogen breakdown. Noradrenaline can function similarly but its major role in the body is as a neurotransmitter in the sympathetic nervous system.

1.07.02.: Chromaffin Granules

The catecholamines are stored intracellularly in membrane-bounded vesicles of mean diameter about 300nm, known as "chromaffin granules". Since the development of procedures to purify chromaffin granules on a large scale by differential and density gradient centrifugation (Smith and Winkler, 1967a), it has been possible to investigate the components of both the chromaffin granule membrane and the matrix content.

1.08.: The Chromaffin Granule Matrix

Table 1.01. lists the components of the chromaffin granule matrix.

1.08.01.: The Soluble Proteins of the Chromaffin Granule Matrix

The soluble proteins of the chromaffin granule, with the exception of dopamine β-hydroxylase, were collectively termed chromogranins (Blaschko et al., 1967), and their major component was named chromogranin A (Schneider et al., 1967). The amino acid composition of the low molecular weight chromogranins was found to be similar to that of Chromogranin A (Smith and Winkler,1967b) suggesting that the smaller chromogranins may in fact be proteolytic products of chromogranin A (Winkler, 1976). Three other families of soluble chromaffin granule matrix proteins have since been identified as being immunologically distinct from the chromogranin A family. These are the enkephalin precursors (Gubler, 1982), the chromogranin B family (Fischer-Colbrie and Frischenschlager, 1985; Falkensammer et al., 1985a) and the chromogranin C family (Winkler et al., 1985).

1.08.02.: Chromogranin A Family

Chromogranin A comprises about 40% of the total soluble protein of the chromaffin granule (25% of the total granule protein) (Winkler, 1976). It is an acidic protein (pI=4.9) of around 70 kilodaltons. Amino acid analysis has revealed that chromogranin A is rich in acidic amino acids, especially glutamic acid, and also has a high content of proline (Smith and Winkler, 1967b). The conformation of chromogranin A approaches that of a random coil with a small section of α-helix (Smith and Winkler, 1967b).

In contrast to a recent report that it exists as an integral membrane protein (Settleman et al., 1985a), chromogranin A has been shown to be a soluble protein, by phase separation in Triton X-114 (Pryde and Phillips, 1985). However, chromaffin granule membrane preparations are always contaminated to some degree by secretory and cytoplasmic proteins (Winkler, 1976), as has beeen found with other types of secretory granule (Castle and Palade, 1978).

Antiserum raised against chromogranin A cross-reacts with several smaller soluble proteins, those of the chromogranin A family (Hortnagl et al., 1974; Kilpatrick et al., 1983; Fischer-Colbrie and Frischenschlager, 1985; Settleman et al., 1985b). The suggestion that the smaller chromogranins arise as a result of intragranular protease action on chromogranin A (Winkler, 1976) has been further substantiated by two recent findings; a) only one primary translation product is recognized by antiserum raised against chromogranin A (see Chapter 5; Falkensammer et al., 1985a), and b) two types of protease activity, a trypsin-like protease (Lindberg et al., 1984) and a cobalt stimulated carboxypeptidase (Fricker and Snyder, 1982) have been identified as components of the chromaffin granule. These are most likely responsible for the processing of enkephalin-containing polypeptides, and, presumably, the chromogranins.

Chromogranin A and the chromogranin A family are glycoproteins, the former containing 5.4% carbohydrate, probably entirely O-glycosidically linked mono- and di-sialylated tri- and tetra-saccharides of N-acetylgalactosamine, galactose, and N-acetyl and/or N-glycolyl neuraminic acid (Kiang et al., 1982;

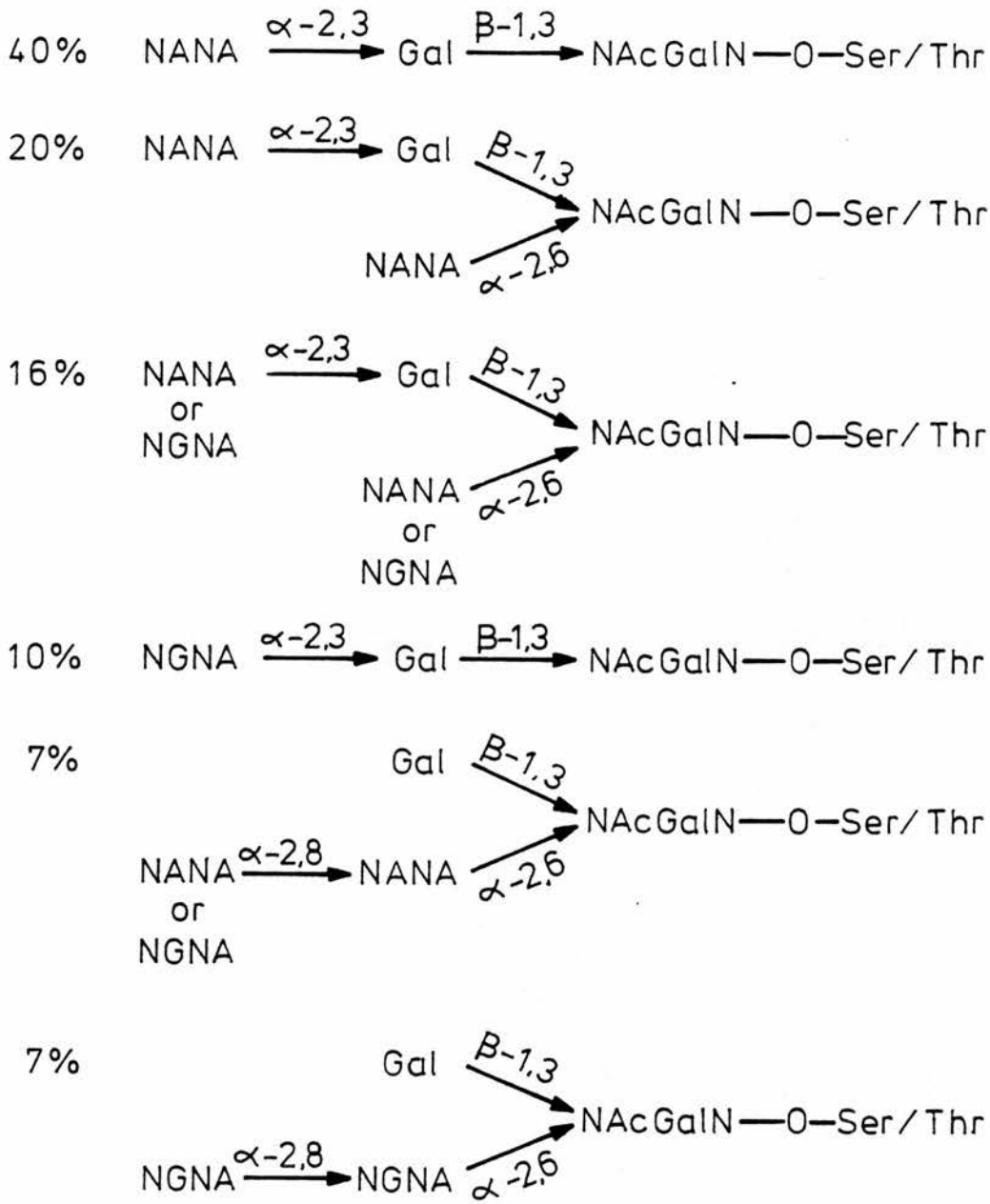


Fig.1.04. THE COMPOSITION OF O-LINKED CARBOHYDRATE CHAINS ON THE CHROMOGRANINS (from Kiang et al., 1982).

Fischer-Colbrie et al., 1982; see Fig.1.04.). The tri- and tetra-saccharides are not uniformly distributed among the chromogranins; tetra-saccharides are concentrated on the smaller chromogranins. At the time of these studies however, members of the chromogranin B and chromogranin C families had not been distinguished from those of the chromogranin A family, so such results must be treated with caution.

1.08.03.: The chromogranin B family

The largest member of this recently identified family has an apparent molecular weight of 100,000 and is slightly less acidic (pI=5.2) than chromogranin A (Fischer-Colbrie and Frischenschlager, 1985). In a similar manner to chromogranin A, antiserum raised against chromogranin B recognises a family of smaller proteins, most likely proteolytic degradation products of chromogranin B (Falkensammer et al., 1985a). A two-dimensional SDS-polyacrylamide gel separation of chromaffin granule soluble proteins resolves the two families but some chromogranins A comigrate with chromogranins B when separated on only a molecular weight basis. On the basis of their lectin binding properties, the chromogranin B family appear to have both N- and O-glycosidically linked oligosaccharides (Apps et al., 1985). Chromogranin B has also been found to be sulphated during its biosynthesis, the post-translational modification which is responsible for the large difference in pI between the primary translation product and the mature protein (Falkensammer et al., 1985b).

Only very recently has the presence of a fourth family of

proteins been discovered within the chromaffin granule matrix, named the chromogranin C family (Winkler et al., 1985). The largest member of this family has a molecular weight of 86,000 and an isoelectric point of around pH5, and was initially identified in the anterior pituitary (Rosa and Zanini, 1983).

1.08.04.:Distribution of the chromogranins

Chromogranin A is not confined to adrenal medullary chromaffin granules. A similar, probably identical protein, has been characterized from the parathyroid gland, parathyroid secretory protein I (Cohn et al., 1982). The first twentyfive amino terminal amino acids of bovine adrenal medullary chromogranin A and bovine parathyroid secretory protein I are identical (Kruggel et al., 1985).

Both proteins are O-glycosylated and immunologically cross-react (Cohn et al., 1982). This discovery stimulated a further search for chromogranin-like proteins outwith adrenergic tissues, and subsequently immunoreactive chromogranins have been found in many polypeptide hormone-storing endocrine glands, albeit at much lower concentrations than in the adrenal medulla (O'Connor, 1983). Immunoreactive chromogranin-like proteins are distributed throughout the nervous system; however they are not co-stored with neurotransmitters and are in fact very prominent in the cytoplasm of brain cells (O'Connor and Frigon, 1984; Somogyi et al., 1984). Exocrine glands, for example the salivary glands, seem to be devoid of chromogranin A-like proteins, as are the amine-storing granules of platelets, thus substantiating the idea that chromogranins are not directly involved with amine storage (Section 1.09.02.). None of

these comparative studies have made any distinction between the three chromogranin families, which we now know are present within the granule matrix. The chromogranins may play a general role in the storage or secretory processes of some endocrine glands. Of the many possible functions that have been proposed for the chromogranins, such as hormones, neurotransmitters or precursors to such molecules, none have been substantiated experimentally. The degree of interspecies homology between chromogranins is surprising since no correlation with any function has been demonstrated (O'Connor et al., 1984).

1.08.05.: Enkephalin-containing peptides

A proenkephalin of 30 kilodaltons has been established as the precursor to several enkephalin-containing peptides of bovine adrenal medulla by cDNA sequencing and by peptide sequencing (Gubler et al., 1982). Proteases, a trypsin-like protease (Lindberg et al., 1984) and a cobalt stimulated carboxypeptidase (Fricker and Snyder, 1982), have been characterized from the chromaffin granule and are probably involved with the processing of proenkephalin to create several enkephalin-containing peptides. Within chromaffin granules, most of the enkephalin sequences occur in proteolytic fragments, the enkephalin-containing peptides. There is very little or no proenkephalin and little free enkephalin. Secreted enkephalin-containing peptides may be more stable than free enkephalins and may also have more potent biological activity (Udenfriend and Kilpatrick, 1983). This extensive intragranular proteolytic processing of proenkephalin is in sharp contrast to that

of chromogranin A which exists primarily in the unprocessed form and is proteolytically processed only very slowly. The extent of processing of chromogranin B is intermediate between that of proenkephalin and that of chromogranin A. The enkephalin-containing peptides are not glycosylated (Kilpatrick et al., 1983b). The role of the medullary enkephalins in vivo is unknown.

1.08.06.: Soluble dopamine β-hydroxylase and soluble glycoprotein III

Both dopamine β-hydroxylase (75 kilodaltons) and an acidic heterogeneous glycoprotein termed glycoprotein III (43 kilodaltons) are found as both soluble and membrane bound forms within the chromaffin granule (Winkler and Carmichael, 1982; Fischer-Colbrie et al., 1984). Both these glycoproteins are minor components of the matrix representing only 2% and 0.25% of the soluble matrix protein respectively. Both are prominent membrane proteins and will be discussed in more detail in sections 1.10.02. and 1.10.04 respectively.

1.08.07.: Proteoglycans

Two proteoglycans containing chondroitin sulphate and dermatan sulphate (proteoglycans I and II) have been identified as soluble components of the chromaffin granule matrix (Kiang et al., 1982). The peptide moiety of proteoglycans I and II are almost identical. It is also of similar amino acid composition but different structure to the chromogranins (Bannerjee and Margolis, 1982). Both proteoglycans contain N- and O-glycosidically linked oligosaccharides

(Kiang et al., 1982), and have a glycosaminoglycan complement which is 48% dermatan sulphate, 23-24% each of chondroitin 4- and chondroitin 6- sulphate and 5% heparan sulphate. They do differ in their relative concentrations of glycosaminoglycan polysaccharide chains, proteoglycan II having twice as many chains as proteoglycan I, and in their O-glycosidically linked tetrasaccharides, proteoglycan I having twice as many as proteoglycan II (Kiang et al., 1982).

1.09.: Small Molecules of the Matrix

1.09.01.: Divalent Cations

The membrane of the chromaffin granule is highly impermeable to divalent cations (Johnson and Scarpa, 1976). Ca^{2+} and Mg^{2+} are however present in the granule matrix.

The total concentration of Ca^{2+} within the chromaffin granule is 20-30mM (Phillips et al., 1977) which accounts for 60% of adrenal medullary Ca^{2+} being stored intragranularly. However, the concentration of free Ca^{2+} within the granule is very low, most of it being bound to ATP and soluble matrix proteins. The uptake of Ca^{2+} into the chromaffin granule is a temperature-dependent, but ATP-independent process, occurring by Ca^{2+}/ Na^{+} exchange, with a stoichiometry of $2Na^{+}$ / Ca^{2+} (Phillips, 1981; Krieger-Brauer and Gratzl, 1982). Whether or not there exists another ATP-stimulated mechanism for the uptake of Ca^{2+} (Burger et al., 1984) is a point of some controversy.

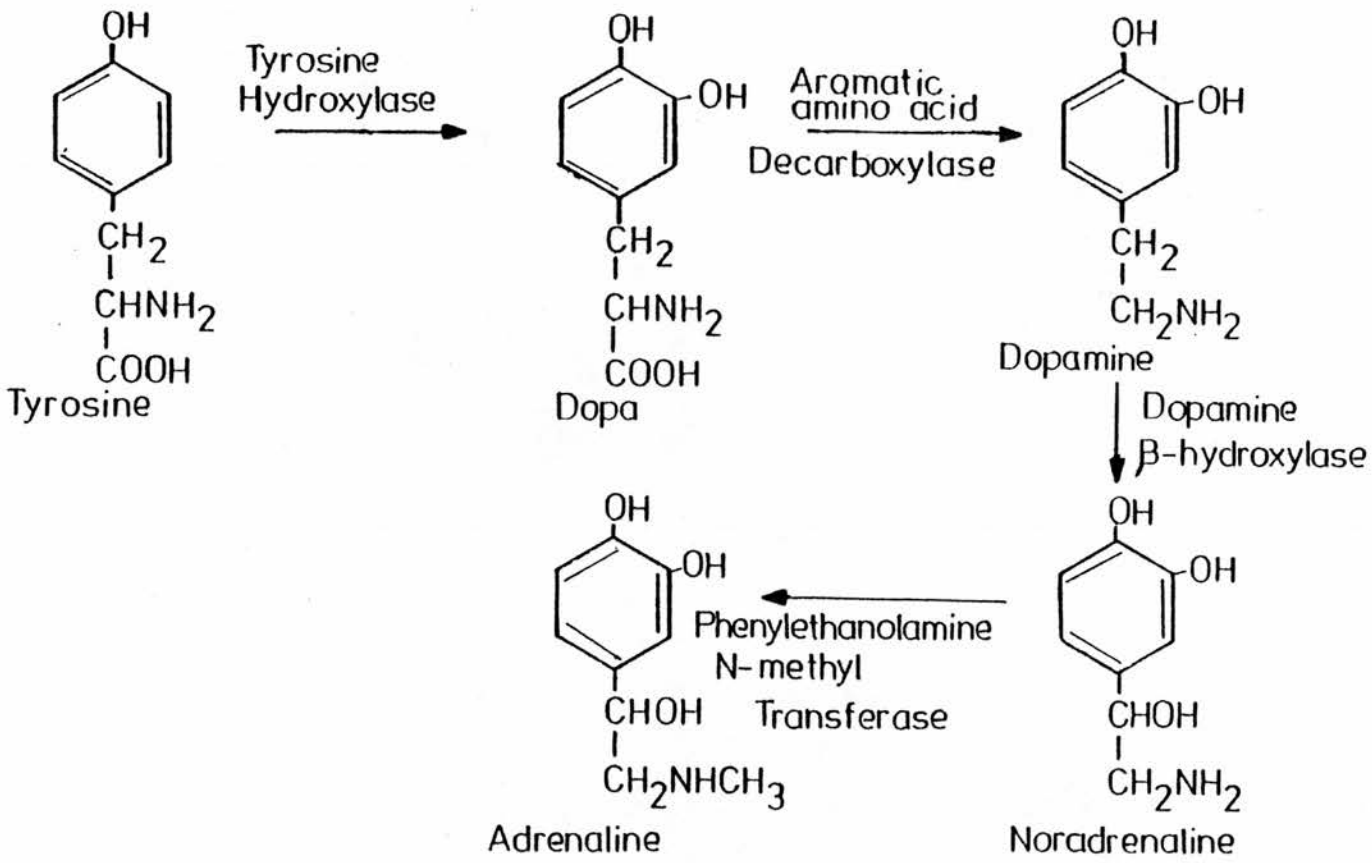


Fig. 1.05. BIOSYNTHESIS OF CATECHOLAMINES

Chromaffin granules may scavenge Ca^{2+} during depolarization of the cell, which is accompanied by an increase in the intracellular calcium concentration (Douglas, 1968). This would present a mechanism for the cell to release accumulated Ca^{2+}.

During the maturation of the chromaffin granule, Ca^{2+} is the first low molecular weight component to accumulate and at this point Ca^{2+} may function to enhance the accumulation of soluble proteins during the process of condensation (Winkler, 1977).

Mg^{2+} is found in the granule matrix at a total concentration of 6mM. Further uptake of Mg^{2+}, which would destabilize the ionic interactions between catecholamine and ATP, (Section 1.09.02) resulting in a loss of catecholamine, may be prevented by the electrochemical gradient across the membrane (Fiedler and Daniels, 1984).

1.09.02.: Catecholamines

Catecholamines are synthesized from tyrosine (Fig.1.05.). Only one of the enzymes involved in catecholamine biosynthesis (dopamine β-hydroxylase) is present within the chromaffin granule. The other enzymes (tyrosine hydroxylase, aromatic L-amino acid decarboxylase and phenylethanolamine-N-methyl transferase) are located in the cytoplasm and are synthesized on free polysomes in contrast to dopamine β-hydroxylase, which is synthesized on bound polysomes (Sabban and Goldstein, 1984).

The total intragranular concentration of catecholamines is about 600mM, in contrast to cytosolic concentrations which are probably in the micromolar range (Phillips, 1982). Catecholamine uptake is mediated by an amine transporter and the process is dependent on a transmembrane pH gradient (ΔpH) and a membrane potential ($\Delta\psi$), generated by the action of an electrogenic, H^+ translocating ATPase (Njus et al., 1981; Apps, 1982). The amine transporter transports dopamine, adrenaline, noradrenaline, serotonin and various analogues of phenylethylamine, and has a higher affinity for the naturally occurring (-) isomers than the (+) stereoisomers (Phillips, 1974). The molecular structure of the carrier is controversial: affinity labelling experiments have suggested components of 45,000 daltons (Gabizon and Schuldinger, 1985) and 70,000 daltons (Isambert and Henry, 1985) involved in amine transport.

Catecholamine uptake occurs slowly and, in vivo, only after the accumulation of Ca^{2+} and nucleotides (Winkler, 1977). Unprotonated catecholamine is thought to be taken up in exchange for a proton (Scherman and Henry, 1981) in an electrogenic process, which therefore depends on both components of the protonmotive force.

The osmotic pressure within the granule is reduced; the concentration of ions within the granule matrix is at least 730mM and yet the granule is isotonic with 300mosM (Winkler, 1976; Winkler and Carmichael, 1982). High molecular weight storage complexes between catecholamines and nucleotides or between catecholamines, nucleotides and soluble protein (particularly the chromogranins) have been ruled

out, mainly by nuclear magnetic resonance data, which suggest that the interior of the granule is fluid and that the catecholamines and protein are free in solution (Sharp and Sen, 1978). Although there is a constant ratio of catecholamines:ATP of about 4:1 within the bovine chromaffin granule matrix, this is different in other species (Winkler and Carmichael, 1982). Kopell and Westhead (1982) concluded that the osmotic pressure of concentrated solutions of catecholamines and ATP spontaneously decreases and that neither protein nor divalent cations are required to achieve the lowered osmotic pressure. It is, however, highly likely that there are interactions between the acidic side chains of the amino acids of the chromogranins and the catecholamines and nucleotides.

Leakage of catecholamines from the granule matrix into the cytoplasm is of physiological significance since noradrenaline, which is formed within the granule, must be converted to adrenaline by the cytosolically located enzyme, phenylethanolamine-N-methyl transferase (Winkler, 1977). This exchange may take place through the action of the catecholamine carrier, or by diffusion through the membrane.

1.09.03.: Nucleotides

The concentration of nucleotides within the chromaffin granule is 220mM of which 75% is ATP, but ADP, AMP, GTP, and UTP are also present (Winkler and Carmichael, 1982). Nucleotide uptake appears to be dependent on the electrical part of an electrochemical proton gradient generated by a proton-translocating ATP'ase, and occurs via a nucleotide translocator (Aberer et al., 1978; Weber et al., 1983).

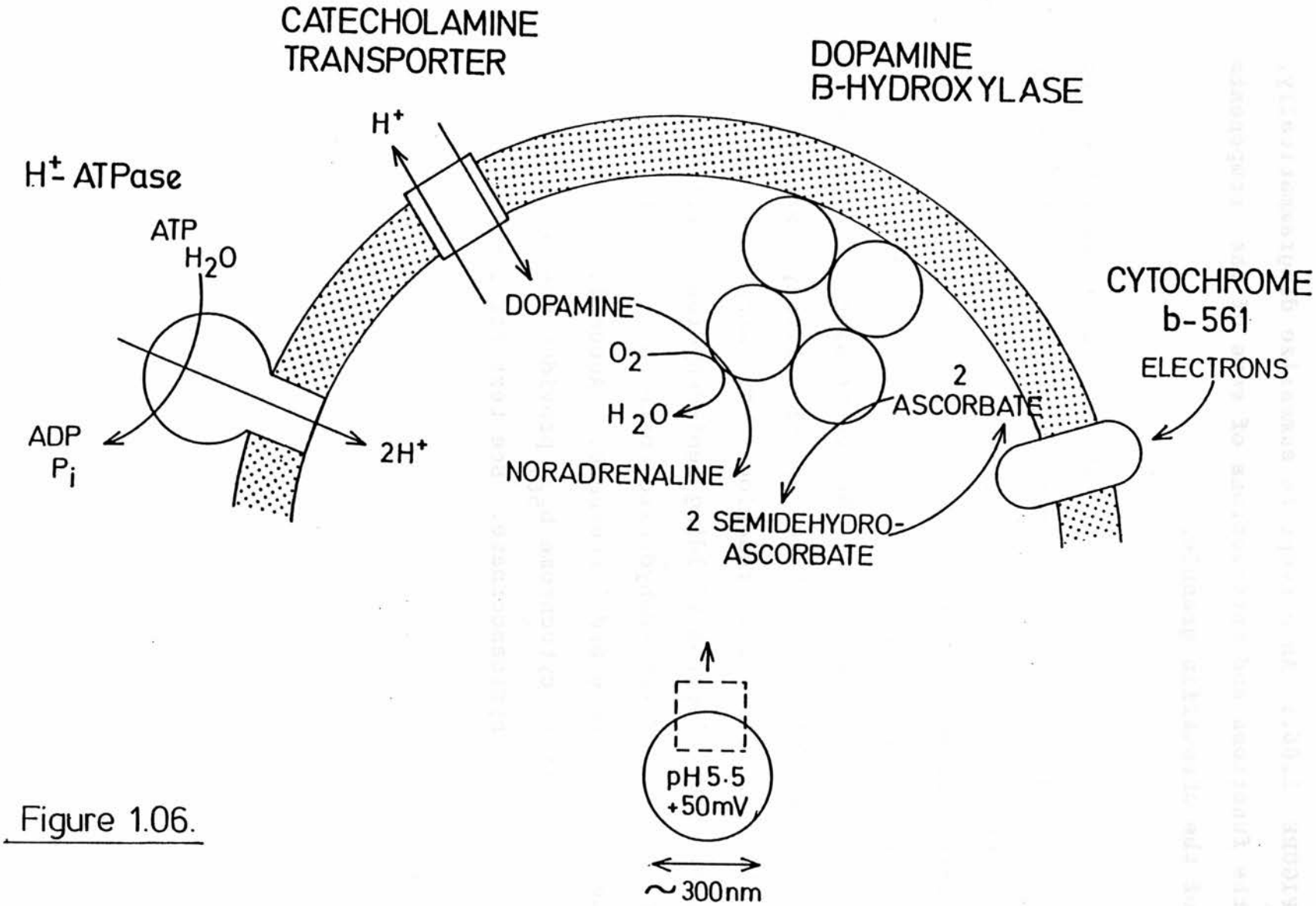

CATECHOLAMINE
TRANSPORTER
DOPAMINE
B-HYDROXYLASE
H+
H+- ATPase
ATP
H2O
DOPAMINE
CYTOCHROME
b-561
O2
ELECTRONS
2
ASCORBATE
H2O
ADP
Pi
2H+
NORADRENALINE
2 SEMIDEHYDRO-
ASCORBATE
pH 5.5
+50mV
~300nm

Figure 1.06.

FIGURE 1.06.: An attempt to summarize diagrammatically, the functions and interactions of some of the components of the chromaffin granule.

The proton-translocating ATPase is responsible for maintaining the acidity and positive potential within the chromaffin granule matrix. During the biosynthesis of catecholamines, dopamine is translocated across the granule membrane in exchange for a proton. Dopamine β-hydroxylase catalyses the conversion of dopamine to noradrenaline. Ascorbate is the likely source of electrons for this reaction. Two ascorbates may each donate one electron yielding semi-dehydroascorbate. Two molecules of semi-dehydroascorbate may then dismutate to dehydroascorbate and ascorbate. Accumulating evidence suggests that cytochrome b_{561} provides the electrons to re-reduce dehydroascorbate. See text for details.

1.09.04.: Ascorbate

Dopamine β-hydroxylase (EC 1.14.17.1) is a mixed function oxidase requiring an electron donor in order to catalyse the conversion of dopamine to noradrenaline (Section 1.10.02.). The best electron donor for soluble dopamine β-hydroxylase *in vitro* is ascorbate.

Ascorbate is present within the chromaffin granule matrix (Terland and Flatmark, 1975) at a concentration of around 20mM (Ingebretson *et al.*, 1980). The chromaffin granule membrane is impermeable to ascorbate, but permeable to dehydroascorbate (Tirrell and Westhead, 1979). Dehydroascorbate presumably diffuses across the membrane to be reduced within the chromaffin granule matrix (Tirrell and Westhead, 1979), possibly by the granule's electron transport system (Section 1.10.03.).

Dopamine β-hydroxylase accepts one electron from ascorbate, thus generating semidehydroascorbate which may dismutate to ascorbate and dehydroascorbate (Skotland and Ljones, 1980). Alternatively the semidehydroascorbate may be re-reduced to ascorbate by electron transport through the cytochrome (See Fig. 1.06.)

However, whether ascorbate is the reductant for dopamine β-hydroxylase *in vivo* remains uncertain since it is apparently unable to reduce the membrane bound form of the enzyme (Grouselle and Phillips, 1982).

1.10.: The Chromaffin Granule Membrane

The membrane of the chromaffin granule has two major functions 1) to transport and accumulate small molecules and 2) to interact with the plasma membrane during exocytosis.

1.10.01.: Membrane Proteins

Approximately 20% of the granule's protein is membrane bound. The topology of the membrane proteins has been studied using impermeant labelling reagents and proteases coupled with SDS-polyacrylamide gel electrophoresis (Huber et al., 1979; Abbs and Phillips, 1980). Most proteins are exposed on the cytoplasmic face, but carbohydrate moieties are exposed only to the granule matrix. Subsequently, the granule matrix proteins were characterized with respect to their lectin binding properties (Gavine et al., 1984) and also by their distribution in various phases of Triton X-114 (Pryde and Phillips, 1985). Some 24 proteins have been characterized by this latter technique in contrast to previous reports of there being between 40-60 membrane proteins Abbs and Phillips, 1980). This earlier over-estimate was probably due to contamination of soluble proteins and microsomal and mitochondrial membrane contamination of the chromaffin granule membrane preparation. The latter has now been reduced by pelleting chromaffin granule membranes through a 1M sucrose cushion after granule lysis (see section 2.01.02.). By far the most abundant proteins are dopamine β-hydroxylase, comprising 20-25% of the membrane protein, and cytochrome b_{561}, comprising

TABLE 1.02.

Proteins known to be present on the chromaffin granule membrane

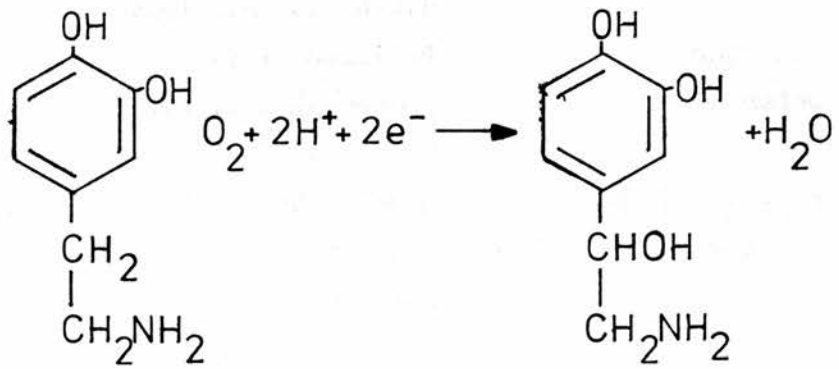
Dopamine β-hydroxylase EC 1.14.17.1	Winkler and Carmichael, 1982*
Cytochrome b561	Winkler and Westhead, 1980*
ATPase I (H^+-translocating) EC 3.6.1.3.	Apps et al., 1980, Percy et al., 1985
Actin	Winkler and Westhead, 1980*
α-actinin	Bader and Aunis, 1983
Spectrin	Aunis and Perrin, 1984
Calmodulin-binding proteins(2)	Bugoyne and Geisow, 1981; Hikita et al., 1984
Catecholamine Transporter	Phillips, 1974
Nucleotide Transporter	Aberer et al., 1978
Ca^{2+}/Na^+ Transporter	Phillips, 1981
Carboxypeptidase B-like enzyme	Supattapone et al., 1984
Phosphatidylinositol kinase EC 2.7.1.67	Winkler and Westhead, 1980*
Glycoprotein II	Fischer-Colbrie et al., 1982; Gavine et al., 1984
Glycoprotein III	Fischer-Colbrie et al., 1982; 1984 Gavine et al., 1984
Glycoprotein IV	Pryde and Phillips, 1985
Glycoprotein J	Abbs and Phillips, 1980; Wood et al., 1985
Glycoprotein K	Abbs and Phillips, 1980; Wood et al., 1985
Sv2	Buckley and Kelly, 1985
Synaptin	Bock and Helle, 1977
65,000 dalton synaptic vesicle protein	Matthew et al., 1981
Adenylate cyclase(?)	Winkler and Westhead, 1980*
Lysophospholipase(?)	Franson and Van den Bosch, 1982

* reviews

15-20%. (Winkler and Westhead, 1980). A list of the proteins, known to be present on the membrane of the chromaffin granule, is given in Table 1.02.

1.10.02.: Dopamine β-hydroxylase

Dopamine β-hydroxylase (EC 1.14.17.1) is the only enzyme involved with catecholamine biosynthesis which is located within the chromaffin granule. It is a copper-containing mixed function oxidase and catalyses the conversion of dopamine to noradrenaline.



Dopamine β-hydroxylase is a glycoprotein of 300,000 daltons and comprises four similar subunits, each of 75,000 daltons. In the presence of chaotropic agents, it is dissociated into dimers of 150,000, and disulphide bond reduction converts it into monomers. The carbohydrate content is 5% and appears to be N-linked to each of the four subunits of dopamine β-hydroxylase as revealed by carbohydrate analysis (Fischer-Colbrie et al., 1982; Margolis et al., 1984) and lectin binding properties (Gavine et al., 1984). Each of the four subunits however does not contain the same carbohydrate moieties (Margolis et al., 1984) and it has been proposed that two of

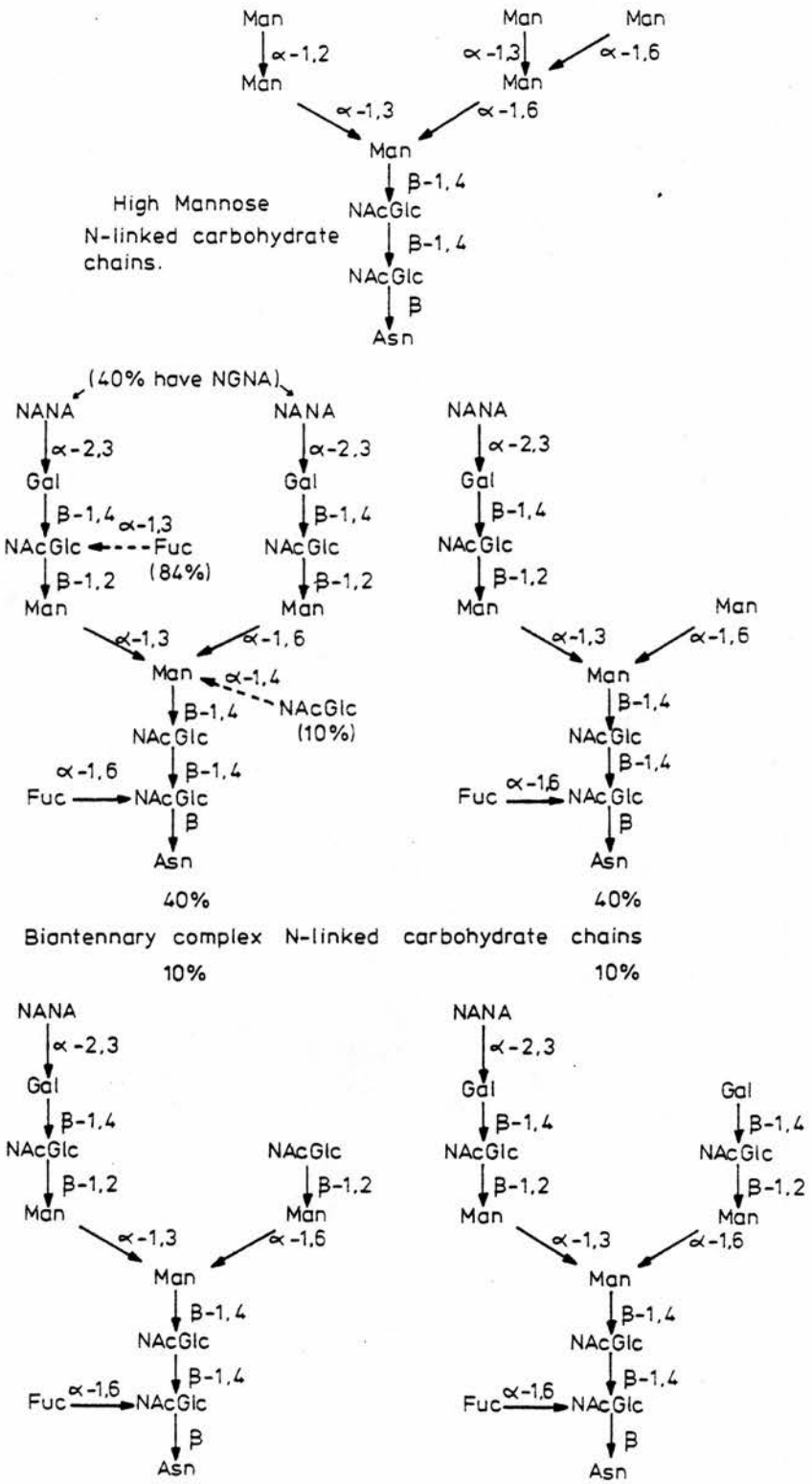


Fig.1.07. The composition of the N-linked carbohydrate chains on DBH (from Margolis et al., 1984).

the subunits contain high-mannose oligosaccharides and that biantennary complex oligosaccharides may be distributed on each of the four subunits or restricted to two subunits (Margolis et al., 1984; see Fig.: 1.07.). There are two different types of polypeptide chain, at least in that one chain has an amino terminal tripeptide extension (Skotland et al., 1977). Whether there are more extensive differences between the chains is at present unclear, but the number of tryptic fragments derived from dopamine β-hydroxylase strongly suggests subunit heterogeneity (Sokoloff et al., 1985).

The membrane bound form of dopamine β-hydroxylase is thought to be attached to the inner face of the chromaffin granule membrane thus exposing the carbohydrate moieties to the matrix. Labelling studies suggest that the membrane bound form of dopamine β-hydroxylase does not span the membrane (Abbs and Phillips, 1980; Duong and Fleming, 1984). So far it has proved difficult to determine the structural difference(s) between the membrane bound and soluble forms of the enzyme since they cross-react immunologically and co-migrate on SDS-polyacrylamide gels (Winkler, 1976). However, the membrane bound form is amphiphilic and can be separated from the soluble form by its detergent binding properties coupled with crossed immunoelectrophoresis (Bjerrum et al., 1979; Skotland and Flatmark, 1979). The two forms of dopamine β-hydroxylase can also be separated by Triton X-114 phase separation, the membrane bound form being found solely in the detergent phase (Pryde and Phillips, 1985). The structural component responsible for the amphiphilicity of the membrane bound form remains unknown. It may be attributable to a hydrophobic sequence of amino acids anchoring it into the membrane,

although protease digests have not revealed any differences between the soluble and membrane bound forms (Sokoloff et al., 1985). The difference may be too small to be detectable. Alternatively, post-translational fatty-acylation could be responsible for anchorage, although it has been reported that newly synthesized dopamine β-hydroxylase from rat pheochromocytoma is not labelled with ^{3}H-palmitic acid (McHugh et al., 1985).

Approximately 60% of dopamine β-hydroxylase is membrane bound in bovine chromaffin granules (Winkler and Westhead, 1980) but this may vary in different species (Phillips, 1982). Different forms of the enzyme may be present in the noradrenaline and the adrenaline storing chromaffin cells, which in turn vary between species (Winkler and Carmichael, 1982). Soluble dopamine β-hydroxylase is resistant to endogenous granule matrix proteases and to digestion by trypsin and chymotrypsin (Helle et al., 1977) in contrast to the other matrix proteins. Resistance may be conferred by the N-linked oligosaccharide chains (Olden et al., 1982).

Dopamine β-hydroxylase is also found within the catecholamine storing vesicles of the sympathetic nervous system. (Klein et al., 1977).

1.10.03.: Cytochrome b_{561}

The second most abundant protein of the chromaffin granule membrane was originally named chromomembrin B. However, it was

subsequently identified by 2-dimensional gel electrophoresis, as being identical to cytochrome b_{561} (Apps et al., 1980). Cytochrome b_{561} has an isoelectric point of 6.2 and its apparent molecular weight varies between 22,000 daltons (Abbs and Phillips, 1980), and 30,000 daltons (Duong and Fleming, 1982) on SDS-polyacrylamide gels and was calculated as 20,500 daltons by analytical ultracentrifugation (Flatmark and Gronberg, 1981). Cytochrome b_{561} appears to be unglycosylated on the basis of its failure to bind lectins (Gavine et al., 1984). Cytochrome b_{561} is a haem-containing protein with a midpoint redox potential of +140mV at pH 7.0 (Flatmark and Terland, 1971).

The function of cytochrome b_{561} is not fully understood but it may be involved with the conduction of electrons to the inner granule membrane surface, which may be required for the regeneration of ascorbate from semidehydroascorbate. Ascorbate is likely to provide the reducing equivalents necessary for the reaction catalysed by dopamine β-hydroxylase (Section 1.10.02.). That chromaffin granule membranes do in fact contain a transmembrane electron carrier, has been demonstrated using chromaffin granule ghosts (Phillips, 1974), in which electron transfer from internal ascorbate to external electron acceptors such as ferricyanide and ferricytochrome c has been shown (Njus et al., 1983; Harnadek et al., 1985). It is very likely that cytochrome b_{561} mediates this transfer of electrons (Srivastava et al., 1984).

Amino acid analysis of cytochrome b_{561} revealed that only 60% of the amino acids are non polar, suggesting that the protein is not

very hydrophobic (Duong and Fleming, 1982). Four cysteine residues were also found, suggesting the possibility of two intrachain disulphide bonds. The amino terminal amino acid is blocked, possibly due to acylation (Duong and Fleming, 1982).

The topology of cytochrome b_{561} in the membrane has been studied using radiolabelled membrane-permeable and membrane-impermeable labelling reagents in combination with immunoprecipitation (Duong and Fleming, 1984) and suggests that cytochrome b_{561} is a transmembrane protein with a large cytoplasmic domain. This contrasts with previous results which suggested that the cytochrome had only a small cytoplasmic domain containing the main antigenic determinants, since protease treatment of intact granules resulted in the cytochrome being reduced in apparent molecular weight by about 1,500 daltons (Abbs and Phillips, 1980; Hunter et al., 1982). There is some evidence from preliminary chemical cross-linking studies that cytochrome b_{561} exists as an oligomer of 4-6 polypeptides in the membrane (Apps et al., 1984).

Cytochrome b_{561} has been identified in neurosecretory granules, not involved in catecholamine biosynthesis, but which contain an ascorbate dependent enzyme, peptidyl α-amidase, a mixed function oxidase with electron supply requirements similar to those of dopamine β-hydroxylase (Duong et al., 1984). This lends support to the hypothesis that cytochrome b_{561} regenerates ascorbic acid, which provides electrons to such enzymes.

1.10.04.: Glycoproteins

The carbohydrate moieties of chromaffin granules proteins are predominantly N-glycosidically linked (Geissler et al., 1977). Despite detailed studies of the glycoproteins in terms of their lectin binding properties (Cahill and Morris, 1979; Gavine et al., 1984), their functions remain obscure. However, large-scale purification of these proteins using Triton X-114 (Pryde and Phillips, 1985) may, in the future, aid further characterization and elucidation of their roles. In the meantime, two major glycoproteins have been given some attention, glycoprotein II and glycoprotein III (Huber et al., 1979).

Glycoprotein III, like dopamine β-hydroxylase, exists in soluble and membrane-bound forms (Fischer-Colbrie et al., 1984). It has an apparent molecular weight of around 43,000 daltons and contains 30% carbohydrate, mainly mannose, galactose. N-acetylgalactosamine and sialic acid (Fischer-Colbrie et al., 1982). Glycoprotein III is very heterogeneous with respect to pI. This heterogeneity can however be reduced by neuraminidase treatment which removes sialic acid residues (Gavine et al., 1984). Glycoprotein III has been identified in the anterior and posterior pituitary (Fischer-Colbrie et al., 1984) but the function of this glycoprotein remains unknown.

Glycoprotein II is also characterized by its high carbohydrate content of 25% which is of similar composition to that of

glycoprotein III. Glycoprotein II has an apparent molecular weight of between 84 and 100 kilodaltons on one-dimensional SDS-polyacrylamide gels (Huber et al., 1979). It has recently been identified on two-dimensional gels as an acidic polypeptide showing a marked degree of heterogeneity in pI (Pryde and Phillips, 1985). However, despite having similar gel characteristics to the Sv2 glycoprotein, a component of neuronal and endocrine tissue secretory granule membranes (Buckley and Kelly, 1985), glycoprotein II has recently been shown to be distinct from this protein. (J.G. Pryde, to be published) .

1.10.05.: ATPase

The acidic interior (pH5.5) and the membrane potential (+50mV inside) of the chromaffin granule are created by a H^+ translocating ATPase (Njus et al., 1981). Inward translocation of protons is coupled to the hydrolysis of ATP (possibly $2H^+$/ATP) and the process is electrogenic since there are no compensatory ion movements.

The H^+ translocating ATPase (ATPase I) exhibits some similarities to the mitochondrial F_1F_0 ATPase, although it differs in subunit composition. ATPase I is very hydrophobic as determined by its distribution after Triton X-114 phase separation and 5 non-glycosylated polypeptides (with apparent molecular weights 70,000, 57,000, 41,000, 33,000 and 16,000 daltons) co-purify with the ATPase activity although only two of the polypeptides (70,000 and 16,000 dalton) are clearly identifiable as components of the ATPase I complex (Percy et al., 1985).

A second ATPase activity (ATPase II) which is not involved with H^+ translocation has also been tentatively identified from chromaffin granule membranes (Apps et al., 1983). It is sensitive to different inhibitors from ATPase I and its sensitivity to vanadate suggests that it may be of the E_1E_2 type ATPase. However it has not as yet been purified. Mitochondrial contamination presents one major problem in the characterization of chromaffin granule membrane ATPase activities. However, solubilization and fractionation of the ATP'ase activities associated with the chromaffin granule membrane in Triton X-114 shows clearly that the mitochondrial F_1 subunits are not involved with the proton-translocating ATPase activity of the membrane (Percy et al., 1985; Pryde and Phillips, 1985).

1.10.06.: Chromaffin Granule Membrane Lipids

The lipid composition of the chromaffin granule membrane (reviewed in Winkler and Carmichael, 1982; Winkler, 1976) is distinguished from that of other intracellular organelles by its high ratio of cholesterol to lipid and by a large proportion (20%) lysophosphatidyl choline (Blaschko et al., 1967b). This high proportion of lysophosphatidylcholine was once thought to be important for promoting membrane fusion during exocytosis, but it has not been found characteristic of secretory granule membranes in general. Most of the lysophosphatidyl choline (70%) is located on the inner leaflet of the lipid bilayer as are most of the gangliosides. Phosphatidylinositol, which becomes phosphorylated by phosphatidylinositol kinase, and phosphatidyl serine are more

abundant on the outer leaflet of the lipid bilayer (Buckland et al., 1978), thus contributing to the negative charge on the granule outer surface.

1.11.: Biogenesis of Chromaffin Granules

As discussed in the review by Winkler (1977), essentially three methods have been used to obtain evidence for the biogenesis of chromaffin granules. Firstly, by perfusing bovine adrenal glands with ^{3}H-leucine, ^{3}H-fucose, ^{32}P-phosphate or ^{35}S-sulphate, the distribution of newly synthesized protein, N-linked glycoproteins (primarily dopamine β-hydroxylase), phospholipids or mucopolysaccharides respectively, could be determined by subcellular fractionation (Winkler et al., 1972; Baumgartner et al., 1973). The distribution of labelled molecules was dependent on the time between pulse-labelling and cell fractionation. Newly synthesized protein was found in the microsomal fraction after a short pulse, but a longer pulse resulted in the redistribution of labelled protein to the soluble fraction of the less-dense, "immature" chromaffin granules. These proteins were identified as chromogranins by SDS-polyacrylamide gel electrophoresis (Winkler et al., 1972). An autoradiographic study using mouse adrenal medulla (Coupland and Kobayashi, 1976) demonstrated the passage of newly synthesized, radioactive polypeptides through the Golgi stack before appearing in immature granules. The transport of the newly synthesized proteins is relatively quick, about 30 minutes (Coupland and Kobayashi, 1976; reviewed in Trifaro and Poisner, 1982). Both the route and the time taken by the secretory proteins of the chromaffin granule are

consistent with that of secretory proteins from other secretory tissues (Palade, 1975). The precursors to N-linked glycoproteins and mucopolysaccharides are incorporated in the Golgi region. Newly-synthesized N-linked glycoproteins and mucopolysaccharides had a similar distribution to newly synthesized protein. Chromaffin granule membranes did not incorporate significant amounts of radiolabelled precursors to protein, glycoprotein or lipid (Winkler et al., 1972), indicating a low turnover of the granule membrane components and suggestive of membrane reutilization (Winkler, 1977).

A second alternative method for studying the biogenesis of chromaffin granules was to induce the synthesis of new granules by overstimulating chromaffin cells with secretagogues such as insulin, to severely deplete them of their catecholamines (Slotkin and Kirschner, 1973a). The newly-formed granules, which could be isolated by sucrose density gradient centrifugation, lacked ATP and catecholamines. Subsequent accumulation of these small molecules appeared to be the rate-limiting step of chromaffin granule maturation (Slotkin and Kirschner, 1973b).

Morphological studies of the chromaffin cell have also provided some details of the cellular mechanism of chromaffin granule assembly (reviewed by Winkler, 1977). Briefly, the endoplasmic reticulum of catecholamine-depleted cells appears swollen and contains electron-dense material, consistent with secretory protein synthesis. Dense material accumulates in the trans Golgi, prior to the budding and condensation processes, to form "immature" or "prosecretory" granules (Section 1.04.). The maturation of the chromaffin granules

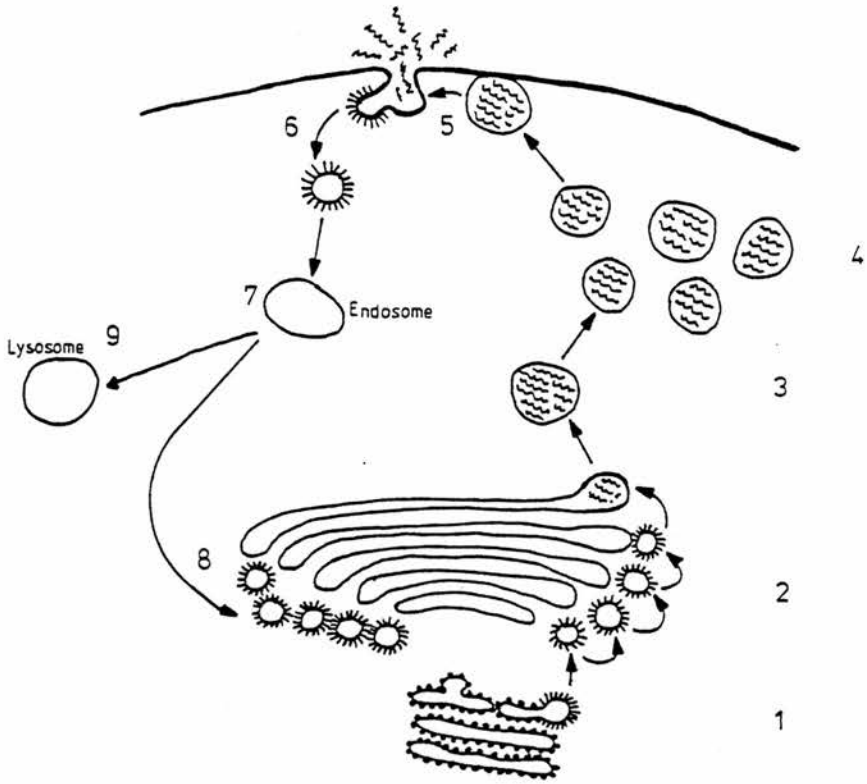


Figure 1.08.

ASSEMBLY OF A CHROMAFFIN GRANULE

1. Proteins are synthesized on polysomes bound to the endoplasmic reticulum.
2. Proteins are transported through the Golgi and packaged within granule membranes.
3. Coated vesicles bud from the trans Golgi and fuse together, giving rise to immature chromaffin granules. Maturation involves the accumulation of small molecules e.g. Ca^{2+}, ATP, catecholamines.
4. Mature granules await the appropriate stimulus for secretion.
5. Exocytosis
6. Endocytosis
7. Reinternalized membranes are "acid washed" within endosomes
8. Granule membranes are returned to the Golgi for reutilization.
9. An unknown proportion of the reinternalized membranes are directed to lysosomes for degradation.

precedes their translocation to an apical region of the cell where, on stimulation, they release their contents in preference to the immature granules. This series of events is analogous to those occurring in other secretory tissues (Palade, 1975) and presumably the general scheme shown in Fig.1.08. can be applied to the biogenesis of chromaffin granules.

As stated previously, kinetic evidence suggests that following discharge of the contents, the chromaffin granule membranes are reutilized (Winkler, 1977). The process of granule membrane retrieval has been demonstrated using thorium dioxide (Nagasawa and Douglas, 1972). The exposure of the chromaffin granule membrane interior on the cell surface, followed by its retrieval has more recently been visualized by fluorescence microscopy in secretagogue-stimulated chromaffin cells using antibodies to dopamine β-hydroxylase and glycoprotein III (Phillips et al., 1983; Patzak et al., 1984). Presumably the reinternalized membranes are "acid washed" in an endosomal compartment prior to their return to the Golgi for reuse, as is thought to occur in other tissues (Farquhar, 1983). The number of times that the secretory granule membrane can be reused before being returned to a lysosome is unknown, as are many of the details of membrane reutilization.

1.12.: Secretion from Chromaffin Cells

Secretion from the adrenal medulla in vivo occurs in response to the binding of acetylcholine to the nicotinic acetylcholine receptors on the chromaffin cell membrane which leads to the opening

of Na^+ channels, the generation of action potentials and the opening of voltage dependent Ca^{2+} channels. The intracellular increase in Ca^{2+} concentration from 0.1μM to 1μM is the signal for secretion (Douglas, 1968), which occurs by exocytosis and results in the extracellular discharge of the chromaffin granule matrix components (Schneider et al., 1967). This process is followed by retrieval (Nagasawa and Douglas, 1972) and reutilization (Winkler, 1977) of the chromaffin granule membranes.

The molecular mechanisms involved with secretion are difficult to study because exocytosis cannot be uncoupled from endocytosis, and the inner surface of the plasma membrane, where these processes are occurring, is rather inaccessible. Response to increased intracellular Ca^{2+} concentrations appears to be effected primarily by calmodulin but the subsequent effects of calmodulin are unknown. Calmodulin's direct involvement with exocytosis was first demonstrated using anti-calmodulin immunoglobulins to inhibit exocytosis of cortical granules from sea urchin eggs (Steinhardt and Alderton, 1982) and, similarly, secretion from stimulated chromaffin cells (Kenigsberg and Trifaro, 1985).

The process of secretion requires Mg^{2+}-ATP (Baker and Knight, 1978). Phosphorylation of certain proteins is enhanced during stimulus-secretion coupling (Amy and Kirschner, 1981) and the sites of phosphorylation have been investigated in vitro (Burgoyne and Geisow, 1981; Burgoyne and Geisow, 1982b; Creutz et al., 1983). Chromaffin granule membrane calmodulin-binding proteins have been identified at high and low Ca^{2+} (Burgoyne and Geisow, 1981; Hikita et

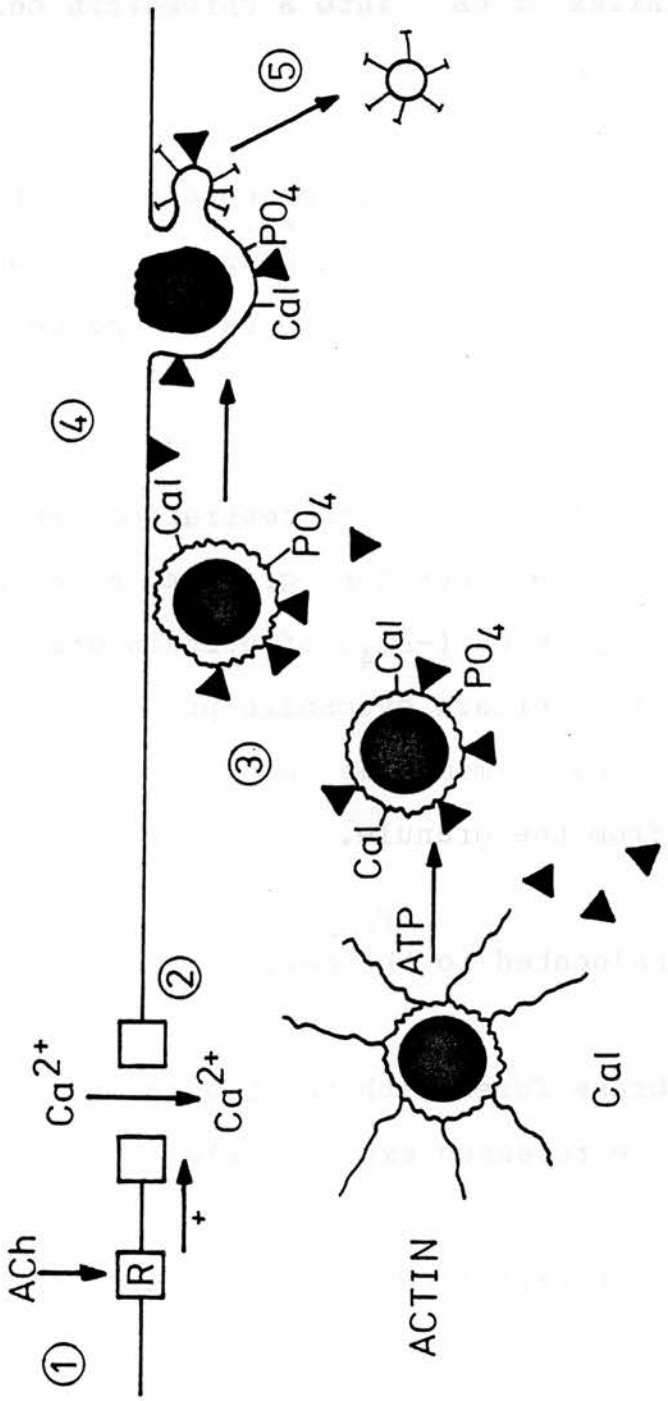


Figure 1.09.

Summary of the events which occur within a chromaffin cell after the influx of Ca^{2+}

(from Burgoyne, 1984)

FIGURE 1.09.: Diagrammatic summary of the events which may occur after the influx of Ca^{2+} into a chromaffin cell (from Burgoyne, 1984)

1. Acetyl choline binds to nicotinic receptors on the surface of the chromaffin cell causing membrane depolarization which in turn opens the Ca^{2+} channels, leading to Ca^{2+} influx.

2. The increased intracellular Ca^{2+} concentration causes (a) calmodulin (cal) to bind certain granule membrane proteins, (b) phosphorylation ($-PO_4$) of certain granule membrane proteins and (c) certain cytosolic proteins (▲) to bind chromaffin granule membrane proteins. Actin filaments dissociate from the granule.

3. The granule is translocated to the cell surface.

4. The granule membrane fuses with the plasma membrane and granule contents are released extracellularly.

5. The membranes are reinternalized and recycled.

al., 1984). Calmodulin-dependent, calmodulin-independent and cAMP-dependent protein kinase activities are associated with the chromaffin granule membrane (Burgoyne and Geisow, 1981; Burgoyne and Geisow 1982a,b; Geisow and Burgoyne, 1983) and some substrates for phosphorylation have been identified as bands on gels. However, as yet there is no direct evidence for the involvement of the phosphoproteins in the process of secretion. Several cytosolic proteins bind reversibly to chromaffin granule membranes in a Ca^{2+}-calmodulin dependent manner including clathrin light chains (Geisow and Burgoyne, 1983) and protein kinase C (Creutz et al., 1983; Summers and Creutz, 1985). The phorbol ester, 12-O-tetradecanoyl phorbol-13-acetate, reduces the Ca^{2+} concentration required to elicit secretion (Knight and Baker, 1983) and several granule membrane proteins have been identified as putative substrates for protein kinase C activity, suggestive of an important role for this enzyme in the process of secretion.

Actin binding may also have a role in secretion since raised Ca^{2+} levels result in the dissociation of bound actin from the chromaffin granule membrane (Pollard et al., 1981). Recently a requirement for a metalloendoprotease has been demonstrated for Ca^{2+} mediated exocytosis but its role is unknown (Mundy and Strittmatter, 1985).

A hypothetical scheme for exocytosis, taken from Burgoyne (1984), is shown in Fig. 1.09. and is based on the assumption that the reactions which occur in vitro also occur in vivo.

CHAPTER 2

MATERIALS AND METHODS

2.01.01.: MATERIALS

Bovine adrenal glands were obtained from a local abbattoir. Dog pancreases and rat livers were gifts from the Cardiovascular Unit, University of Edinburgh.

All common laboratory chemicals and reagents were obtained from BDH, Poole, Dorset, and AnalaR grade reagents were used whenever available.

2.01.02.: Materials used for the preparation of bovine adrenal medullary subcellular fractions

Sucrose, HEPES, EDTA, PMSF, benzamidine HCl, Trizma base, 2-mercaptoethanol, α-methyl-D-mannoside, N-lauroyl sarcosine, diethyl pyrocarbonate, chymotrypsin (bovine pancreas), and papain, type IV (papaya latex) were all obtained from Sigma (London) Chemical Company.

Guanidine thiocyanate (purum grade) was obtained from Fluka A.G., Buchs, Switzerland. Oligodeoxythymidylic acid cellulose was from Bethesda Research Laboratories, Maryland, U.S.A.

Octaethylene glycol dodecylether ($C_{12}E_8$) was obtained from the Kouyoh Trading Co., Tokyo, Japan. Sepharose CL-4B was from Pharmacia, Milton Keynes. U.K. and dithiothreitol was obtained from Boehringer Corporation (London).

2.01.03.: Materials used in translation systems

Phenylhydrazine, glutathione, heparin, haemin (bovine), creatine kinase (rabbit muscle), spermidine phosphate, creatine phosphate (disodium salt), micrococcal nuclease (S. aureus, Foggi strain, Grade VI), EGTA, trypsin (bovine pancreas type III-S), pronase (Streptomyces griseus), soyabean trypsin inhibitor, cycloheximide, tetracaine HCl, collagenase (Clostridium histolyticum) and casein acid hydrolysate (Type I) were all obtained from Sigma (London) Chemical Company.

Threo-β-hydroxy-DL-leucine was obtained from Calbiochem-Behring Corporation, La Jolla, CA 92037.

L-^{35}S-methionine (>800Ci/mmol), L-[4,5]-^{3}H-Leucine (142Ci/mmol) and human placental ribonuclease inhibitor were obtained from Amersham International plc, U.K.

Biogel P-100 was from Bio-Rad Laboratories, Triton X-114 was from Fluka A.G., Buchs, Switzerland, and Nitocellulose membrane filters (pore size 0.45um) were from Schleicher and Schull, Dassel, W. Germany.

Heat-inactivated and formalin-fixed S. aureus cells were a gift from Prof. C. Brown, Dept. of Brewing and Biological Science, Herriot-Watt University, and wheatgerm was a gift from Dr. C. Leaver, Dept. of Botany, University of Edinburgh.

2.01.04.: Materials used for Gel Electrophoresis and related techniques

Acrylamide (Electran grade), N,N' methylene bisacrylamide (Electran grade) and SDS (specially pure) were from BDH, Poole, Dorset. Polyacrylamide (Carboxyl modified-low carboxyl content) was from Aldrich Chemical Company, Gillingham, Dorset. Urea (specially pure) was from Serva and Bio-Lyte ampholines, pH ranges 3-10 and 4-6, (40%w/v), were from BioRad Laboratories. Ampholines, pH range 9-11 were obtained from LKB. Agarose was from Litex, Denmark, and bovine serum albumin (Fraction V) was from Boehringer Corporation (London).

2.01.05.: Materials used in other procedures

DEAE Affi-gel Blue was from BioRad Laboratories and 2,5,diphenyloxazole and 1,4,di-2-(5-phenyloxazolyl) benzene were obtained from Koch Light Laboratories Ltd. Phenyl isothiocyanate was from Sigma (London) Chemical Company and DABITC, trifluoracetic acid and polyamide thin layer chromatography sheets were from BDH, Poole, Dorset.

2.02.: PREPARATION OF MATERIALS FROM THE BOVINE ADRENAL MEDULLA

2.02.01.: Preparation of Chromaffin Granules from the Bovine Adrenal Medulla

Bovine adrenal glands, usually between 40 and 50, were collected as soon as possible after slaughter and transported to the laboratory on ice. Each gland was freed of fat and the adrenal medulla dissected from the cortex. The medullae were placed in about 300ml of ice-cold 0.3M sucrose, 10mM HEPES-NaOH, pH7, and then passed through a stainless steel mincer. The resulting brei was homogenised (10 strokes) in a glass Potter-Elvejhem homogeniser with a loose-fitting teflon pestle. The homogenate was diluted to 1,000ml with buffered sucrose and centrifuged at 1,500 g_{av} (4,000rpm) for 5 minutes at 4^{o}C in a Beckman JA-14 rotor. The pellet of cell debris was discarded. The supernatant was centrifuged at 19,000g_{av} (14,000rpm) for 30 minutes at 4^{o}C in a Beckman JA-14 rotor. The supernatant was discarded. The pellet, containing a bottom layer of pink chromaffin granules surrounded by a fluffy brown layer of mitochondria, could be used to prepare crude or pure chromaffin granules thus:-

1. A crude preparation of chromaffin granules was obtained by washing off the fluffy brown layer of mitochondria with some buffered sucrose. The chromaffin granules were then resuspended by gentle homogenization in buffered sucrose and centrifuged at 19,000g_{av} (14,000 rpm) in a Beckman JA-14 rotor for 20 minutes at 4^{o}C. The supernatant was discarded and any remaining mitochondria washed off

the chromaffin granule pellet. The pink pellet of chromaffin granules was then resuspended in 5-8ml of buffered sucrose.

2. A pure preparation of chromaffin granules was obtained by resuspending the total pellet by gentle homogenisation in about 120ml of buffered sucrose and layering 20ml of this suspension over 50ml of 1.8M sucrose, 10mM HEPES-NaOH, pH7, in 70ml polycarbonate centrifuge tubes. The tubes were then centrifuged at 161,000g_{av} (45,000 rpm) in a Beckman 45Ti rotor for 60 minutes at 4^{o}C. The supernatant was removed by aspiration, the tubes wiped free of adherent material, and the pellet of pure chromaffin granules resuspended in 5-8ml of buffered sucrose.

2.02.02.: Chromaffin Granule Lysate

Pure chromaffin granule pellets (Section 2.02.01) were resuspended by gentle homogenisation in 210ml of 10mM HEPES-NaOH, pH7@0^{o}C. The suspension was centrifuged at 161,000g_{av} (45,000 rpm) in a Beckman 45Ti rotor for 20 minutes at 4^{o}C. The resulting supernatant contained the chromaffin granule matrix proteins (chromaffin granule lysate), and the pellets were crude chromaffin granule membranes. The chromaffin granule lysate was re-centrifuged at 227,000g_{av} (50,000 rpm) in a Beckman 50.2Ti rotor for 60 minutes to remove any residual membrane fragments. The lysate was then dialysed at 4^{o}C against 0.02M sodium phosphate buffer pH7.4 (3 changes; 100volumes), to remove catecholamines.

2.02.03.:DEAE-Cellulose Chromatography of Chromaffin Granule Lysate Proteins

A 40cm x 3.8cm^2 column was packed with DEAE cellulose (Whatman DE-32) equilibrated with 0.02M sodium phosphate buffer, pH7.4. About 300ml of dialysed lysate (1mg protein/ml) was applied to the column and washed on with 50ml 0.02M sodium phosphate buffer, pH7.4. The proteins were eluted with 600ml of sodium phosphate buffer with an increasing salt gradient from 0-0.4M sodium chloride. The flow rate was 0.6ml/minute, and fractions of 6.5ml were collected and their A_{280} monitored (Fig. 3.02.)

2.02.04.: Preparation of Pure Chromaffin Granule Membranes

The chromaffin granule membrane pellets obtained from the centrifugation of lysed granules (Section 2.02.02) were resuspended by homogenisation in about 40ml 10mM HEPES-NaOH, pH7. Two 20ml aliquots were overlayed onto 50ml of 1M sucrose, 10mM HEPES-NaOH, pH7, in 70ml polycarbonate centrifuge tubes and centrifuged at 161,000g_{av} (45,000rpm) in a Beckman 45Ti rotor for 30 minutes at 4 ^{o}C. Granule membranes were collected from the 1M sucrose interface with a Pasteur pipette. Contaminating mitochondrial and microsomal membranes pelleted. The protein concentration of the purified membranes was about 7mg/ml and typically 60-70mg of total protein was collected. Purified membranes were stored at -20^{o}C in 10mM HEPES, NaOH, pH7, 1mM dithiothreitol, 0.1mM EDTA.

2.02.05.: Purification of Cytochrome b_{561} from Chromaffin Granule Membranes

The method used was that of Apps et al., 1980.

The chromaffin granule crude membrane pellet, obtained from the centrifugation of lysed granules (Section 2.02.02.), was resuspended in 20mM HEPES-NaOH, pH7.5. 1mM dithiothreitol, 0.1mM EDTA, at a concentration of about 4mg protein/ml, placed on ice and stirred gently. The proteins were solubilized by the addition of 50µl of 10% (w/v) octaethylene glycol dodecylether ($C_{12}E_8$) per mg of protein. When the membranes were fully solubilized, 10µl of 10% (w/v) sodium cholate, pH7, was added per mg of protein. To the solubilized membranes was added 0.25 volumes of saturated ammonium sulphate, pH7.5, bringing the ammonium sulphate concentration to 0.78M. The membranes were left on ice for 20 minutes, and then centrifuged for 30 minutes at 196,000g_{av} (40,000rpm) in a Beckman SW41 rotor at 4°C. The resulting red supernatant was removed with a Pasteur pipette, leaving behind a floating, white lipid cake. Another 0.25 volumes of saturated ammonium sulphate was added to the supernatant, increasing the ammonium sulphate concentration to 1.29M. The pH was then adjusted to 5.6 with 10%(v/v) acetic acid, and the solution allowed to stand on ice for 20 minutes, before centrifugation for 30 minutes at 196,000g_{av} (40,000rpm) in a Beckman SW41 rotor. The resulting dark red floating viscous layer was carefully removed and diluted with 1 volume of 20mM HEPES-NaOH, pH7, 0.1% $C_{12}E_8$. This was dialysed for 2-3 hours against 100 volumes of 20mM HEPES-NaOH, pH7, followed by further dialysis overnight with 100 volumes of the same buffer. After dialysis, the solution was centrifuged at 50,000g_{av} (30,000rpm)

for 30 minutes in a Beckman 50Ti rotor. The crude cytochrome solution (about 4ml) was then applied to a 30cmx2cm^2 column, packed with 6-aminohexyl CL sepharose 4B (low degree of substitution) which had been equilibrated with 20mM HEPES-NaOH, pH7, 0.01% $C_{12}E_8$. Cytochrome b_{561} was eluted with the same buffer. The flow rate of the column was 18ml/hour and fractions of 1ml were collected. The absorbance of the fractions at 280nm was monitored and once it had fallen to zero, the column was washed with 20mM HEPES-NaOH, pH7, 0.01% $C_{12}E_8$ containing 0.5M sodium chloride. Fractions of the highest purity, as assessed by SDS-polyacrylamide gel electrophoresis, were pooled and lyophilized. The cytochrome was stored at -20^{o}C after lyophilization.

2.02.06.: Preparation of Chymotrypsin- or Papain-Cleaved Cytochrome b_{561}

Crude chromaffin granules were prepared as described in Section 2.02.01.. The pellet of crude chromaffin granules was resuspended in 60ml of 0.3M sucrose, 10mM HEPES-NaOH, pH7. This suspension was then adjusted to a final concentration of 0.1mg/ml in chymotrypsin or papain. When papain was used, the suspension was adjusted to 1mM dithiothreitol to preserve papain in the reduced and active state. The suspension of granules was then gently agitated overnight at room temperature. Chymotrypsin was inhibited by the addition of phenylmethyl sulphonyl fluoride to 1mM and this was subsequently present in all buffers at 0.1mM. Papain was inhibited by the addition of iodoacetamide to 5mM and iodoacetamide was subsequently present in all buffers at 1mM.

After inhibiting the chymotrypsin or papain, the granules were centrifuged at 30,000g_{av} (15,000rpm) in a Beckman JA-20 rotor for 20 minutes at 4 ^{o}C. The pellet was resuspended in 120ml of buffered sucrose containing the appropriate protease inhibitor, and 6x20ml aliquots were overlayed onto 6x50ml of 1.6M sucrose, 10mM HEPES-NaOH, pH7 and pure chromaffin granules were obtained by centrifugation as described in Section 2.02.01. The granules were lysed (Section 2.02.02.) and the resulting crude membrane pellet was resuspended and solubilized (Section 2.02.05.). Protease-cleaved cytochrome b_{561} was then purified by the procedure described in Section 2.02.05.. The final yield of protease cleaved cytochrome b_{561} was usually 12-15mg. A Coomassie blue-stained gel of protease-cleaved cytochrome b_{561} is shown in Fig. 6.02.01.

2.02.07.: Preparation of Reduced and Carboxymethylated Cytochrome b_{561}

Cytochrome b_{561}, purified by chromatography on 6-aminohexyl CL-sepharose 4B, was electroeluted from a 10% SDS-polyacrylamide gel, dialysed against 10% ethanol and lyophilized.

The procedure used for carboxymethylation was that of Pierce et al., 1976. The freeze-dried cytochrome was dissolved in 1ml of 0.1M Tris-HCl, pH8.5, 2.6mM EDTA, 10mM dithiothreitol, 6M urea. Reduction was allowed to proceed overnight, under nitrogen. Alkylation was performed in the absence of light by the addition of 500µl of 0.2M Tris-HCl, pH8.5, 2.6mM EDTA, 50mM iodoacetamide, followed by

incubation on ice for 15 minutes and then for a further 30 minutes at 20°C. The solution of carboxymethylated cytochrome b_{561} was then dialysed against 10mM HEPES-NaOH, pH7.4, (3 changes; 100volumes) 10mM NaCl, and lyophilized.

2.02.08.: Cleavage of Cytochrome b_{561} with Cyanogen Bromide

Cyanogen bromide cleavage was performed according to Gross, 1967.

Freeze-dried, electroeluted cytochrome b_{561} was resuspended in 70% formic acid. A weight of cyanogen bromide, equal to the weight of protein, was dissolved in 70% formic acid, and added to the protein solution. The final volume of the reaction was 100µl. After incubation at room temperature for 24 hours, the reaction was terminated by the addition of 9 volumes of double-distilled water followed by lyophilization. The lyophilized sample was resuspended in 1ml of double-distilled water and again lyophilized. This procedure was repeated twice more to rid the sample of cyanogen bromide.

2.02.09.: Isolation of Bovine Adrenal Medullary RNA

Certain precautions were taken to minimise the risk of ribonuclease contamination. These were:-

1. Glassware was baked at 200°C for 4 hours.
2. Disposable laboratory equipment was treated with 0.1%(v/v) diethylpyrocarbonate for 30 minutes at room temperature and then autoclaved for 20 minutes at 16 psi. Equipment which could not be

autoclaved was washed thoroughly with sterile, double-distilled, deionised water to remove traces of diethylpyrocarbonate.

3. Gloves were worn throughout.

4. All solutions were filtered through nitrocellulose (pore size 0.45μm) and autoclaved wherever possible.

5. Triple glass-distilled, deionised sterile water was used for all solutions required during the preparation or translation of the RNA.

Bovine adrenal medullary RNA was isolated according to Chirgwin et al., 1979, in conjunction with centrifugation through caesium chloride, according to Glisen et al., 1974.

Bovine adrenal glands were collected from a local abbattoir. On collection, about 15-30 minutes after slaughter, the medullae were immediately dissected from the gland, quick frozen in liquid nitrogen, and transported to the laboratory. Twelve grams of frozen adrenal medullae were crushed and transferred to a beaker at room temperature containing 100ml of freshly prepared and millipore-filtered (pore size 0.45μm) guanidine thiocyanate buffer (5M guanidine thiocyanate, 50mM Tris-HCl, pH7.5, 10mM EDTA, 5% 2-mercaptoethanol). N-lauroyl sarcosine was added to a concentration of 4%(w/v) and caesium chloride was added to 0.15g/ml. After mixing, the suspension was thoroughly homogenised (10 strokes up/10 strokes down) in a glass Potter-Elvehjem homogeniser with a loose-fitting pestle. The resulting viscous solution was left at room temperature for 30 minutes before centrifugation in Corex glass tubes at $30,000g_{av}$ (15,000rpm) in a Beckman JA-20 rotor for 20 minutes at $20^{o}C$

to pellet cell debris.

The resulting supernatant was layered onto 2.5ml of 5.7M caesium chloride, 100mM EDTA, in 14ml Beckman polycarbonate "Quick seal" centrifuge tubes and centrifuged for 20 hours at 160,000g_{av} (38,000rpm) in a Beckman 70.1Ti rotor at 20°C. The resulting aqueous layer containing denatured protein was removed with a Pasteur pipette and discarded. The centrifuge tubes were placed on ice, and the top of the caesium chloride cushion and the sides of the tubes were washed thoroughly with water to remove traces of the aqueous layer. The DNA and caesium chloride solution were removed and discarded, revealing a transparent pellet of RNA. The RNA pellets were resuspended in ice-cold 10mM Tris-HCl, pH7.4 to a total volume of 6ml. RNA was precipitated by the addition of 2.5 volumes of cold ethanol and storage for 30 minutes at -70°C, followed by storage for 60 minutes at -20°C. RNA was collected by centrifugation at 30,000g_{av} (15,000rpm) in a Beckman JA-20 rotor for 30 minutes at 4°C. The RNA pellet was resuspended in 10mM Tris-HCl, pH7.4 and precipitated by the addition of sodium chloride to 300mM followed by 2.5 volumes of ethanol and storage overnight at -20°C.

2.02.10.: Isolation of Poly A^+ RNA from Total Bovine Adrenal Medullary RNA

The procedure used was that of Aviv and Leder, 1972, utilizing affinity chromatography.

A jacketed glass column at a constant temperature of 30°C was

used. One gram of oligodeoxythymidylic acid cellulose was packed into the column and washed with low salt buffer (10mM Tris-HCl, pH7.4, 1mM EDTA, 0.2%(w/v) SDS). It was then equilibrated with high salt buffer (0.5M NaCl, 10mM Tris-HCl, pH7.4, 1mM EDTA, 0.5%(w/v) SDS).

The precipitated RNA was centrifuged as before (Section 2.01.09.) and resuspended in 10mM Tris-HCl, pH7.4, 2mM EDTA, 100mM NaCl, 0.5%(w/v) SDS. The suspension was heated for 10 minutes at 65^{o}C to disrupt aggregates. The salt concentration was adjusted to 500mM and the solution was cooled to 30^{o}C prior to being loaded onto the column. The high salt buffer was used to elute the poly A^{-}-RNA. Fractions of 2.5ml were collected into glass test-tubes, and their absorbance at 260nm read. Typically, 70-80ml of high salt buffer were required to elute the poly A^{-}-RNA. The poly A^{+}-RNA was eluted with low salt buffer and 1ml fractions were collected. The fractions with the highest concentrations of poly A^{+}-RNA, as assessed by their absorbance at 260nm, were pooled and adjusted to 300mM NaCl. The RNA was precipitated by the addition of 2.5 volumes of ethanol and storage overnight at -20^{o}C. The oligodeoxythymidylic acid cellulose was washed with 10ml of 0.5M NaOH, followed by 50ml of low salt buffer and stored in 1%SDS.

After centrifugation at 30,000g_{av} (15,000rpm) in a Beckman JA-20 rotor for 30 minutes at 4^{o}C, the poly A^{+}-RNA was resuspended in 300-500ul 10mM Tris-HCl, pH7.4. A 10µl sample was taken and diluted 1:100 with the same buffer. The absorbance of the sample at 235nm, 260nm, and 280nm was read and the A_{260}:A_{280} and the A_{260}:A_{235} ratios

were calculated. Ratios of greater than 2 meant that there was no significant contamination of the RNA with proteins or polysaccharides respectively. A conversion factor of 23 Absorbance units at 260nm for 1mg of RNA was used to calculate the recovery of RNA. Typically, about 350µg of poly A^+-RNA was recovered from 12g of adrenal medullae.

2.02.11.: Storage and Preparation of Poly A^+-RNA for Translation

Poly A^+ RNA was stored at $-70^{O}C$ in a 70%(v/v) ethanol suspension containing 0.3M NaCl. Before use in translation reactions, a known amount of RNA was removed and collected by centrifugation in a MSE microfuge for 4 minutes at 11,800g. The RNA was resuspended to a concentration of 1µg/µl in sterile water and heated at $65^{O}C$ for 10 minutes to disrupt aggregates. The RNA was cooled on ice prior to its addition to translation reactions.

2.02.12.: Preparation of Bound Polysomes from Bovine Adrenal Medullae

Separation and isolation of bound polysomes has been most thoroughly studied using rat liver and cultured mouse myeloma cells. The method used to isolate bound polysomes from bovine adrenal medullae was a slightly modified version of the procedure used by Gaetani et al., 1983, to isolate translatable membrane-bound polysomes from rat liver.

Adrenal medullae from about 40 glands were dissected, minced and homogenised in 0.3M sucrose, 10mM HEPES-NaOH, pH7, 3mM dithiothreitol in a motor-driven Potter-Elvejhem glass homogeniser with a loose-fitting teflon pestle. The final volume of the homogenate was adjusted to 400ml and centrifuged at $1,500g_{av}$ (4,000rpm) in a Beckman JA-14 rotor for 5 minutes at $4^{o}C$. The resulting supernatant was centrifuged at $19,000g_{av}$ (14,000rpm) in a Beckman JA-14 rotor at $4^{o}C$. Approximately half of the initial volume was recovered as the post-mitochondrial supernatant and this was made 10%(v/v) in rat liver high speed supernatant (Section 2.03.15.). Discontinuous sucrose gradients were prepared using rat liver post-mitochondrial supernatant and 2.5M sucrose to create fractions of various sucrose densities. Post-mitochondrial supernatant in 1.8M sucrose, 1.55M sucrose and 1.35M sucrose were prepared in 70ml polycarbonate centrifuge tubes. The discontinuous gradient consisted of 13ml of 1.8M sucrose/post-mitochondrial supernatant, 8ml of 1.55M sucrose/post-mitochondrial supernatant, 46ml of 1.35M sucrose/post-mitochondrial supernatant and this was overlayed with 1M sucrose. The gradients were centrifuged for 8 hours at $161,000g_{av}$

(45,000rpm) in a Beckman 45Ti rotor at 4°C. Smooth microsomes banded at the 1M/1.35M sucrose interface and crude heavy rough microsomes banded at near the 1.55M/1.8M sucrose interface. Rough microsomes were removed using a pump and were diluted with 1 volume of 100mM Tris-HCl, pH7.4, 100mM KCl, 20mM $MgCl_2$, 10%(v/v) rat liver P-100 fraction (Section 2.03.15.). The rough microsomes were solubilized by the addition of sodium deoxycholate and Triton X-100 to concentrations of 0.5%(w/v) and 1%(w/v). This solution was overlayed in 12ml polyallomer centrifuge tubes containing a discontinuous step gradient of 5ml 2M sucrose, 50mM Tris-HCl, pH7.4, 50mM KCl, 20mM $MgCl_2$, 3mM dithiothreitol and 3ml 1.5M sucrose, 50mM Tris-HCl, pH7.4, 50mM KCl, 20mM $MgCl_2$, made 25%(v/v) in the solubilized rough microsomes. The gradients were centrifuged at 170,000g_{av} (43,000rpm) in a Beckman 50.2Ti rotor for 12 hours at 4°C. The resulting pellet of bound polysomes was washed twice with 50mM Tris-HCl, pH7.4, 50mM KCl, 5mM $MgCl_2$, and resuspended in 300µl of the same buffer with 100 units of human placental ribonuclease inhibitor. Aliquots of 20µl were quick frozen in liquid nitrogen and stored at -70°C.

2.02.13.: Preparation of Adrenal Medullary Rough Microsomes

The procedure for the preparation of bound polysomes (Section 2.02.12) was followed up to the removal of rough microsomes from the 1.8M sucrose/1.55M sucrose interface and rough microsomes were prepared as described by Gaetani et al., 1983.

The microsomes taken from this interface, were diluted with 2 volumes of 50mM Tris-HCl, pH7.4, 50mM KCl, 5mM $MgCl_2$, containing

10%(v/v) P-100 fraction (Section 2.03.16). The rough microsomes were layered onto discontinuous sucrose gradients of 1ml 1.8M sucrose Tris-K^+-Mg^{2+}, containing 10%(v/v) P-100, 1ml 1.35M sucrose Tris-K^+-Mg^{2+}, containing 20%(v/v) P-100, and 2ml of 0.7M sucrose, Tris-K^+-Mg^{2+}, containing 10%(v/v) P-100in 12ml polyallomer centrifuge tubes. The gradients were centrifuged at 196,000g_{av} (40,000rpm) for 90 minutes in a Beckman SW41 rotor at 4°C. Concentrated rough microsomes were carefully removed from the 1.8M sucrose/1.35M sucrose interface. They were diluted with Tris-K^+-Mg^{2+}, containing 20%(v/v) P-100, and further concentrated by centrifugation through another discontinuous sucrose gradient of 1.5ml of 1.8M sucrose Tris-K^+-Mg^{2+}, containing 10%(v/v) P-100, and 1.5ml of 1M sucrose Tris-K^+-Mg^{2+}, containing 20%(v/v) P-100, in 12ml polyallomer tubes. The gradients were centrifuged for 60 minutes at 196,000g_{av} (40,000rpm) in a Beckman SW41 rotor at 4°C. The concentrated rough microsomes were removed from the 1M sucrose/1.8M sucrose interface. The A_{260}:A_{280} ratio was determined in 0.2%(w/v) SDS. The rough microsomes were used immediately in translation reactions.

2.02.14.: Isolation of Chromaffin Cells

Cells were isolated as described by Patzak et al., 1984. Bovine glands were freed of fat and perfused retrogradely for 10 minutes with aerated Krebs-Henselheit buffer at 37°C. Collagenase (0.05% w/v) was added to fresh buffer and the gland was perfused with this solution for 30 minutes at 37°C. The medulla was dissected from

the cortex, finely chopped with scissors, and incubated for 15-20 minutes in a fresh collagenase solution. The cell suspension was filtered through nylon mesh and washed twice, by centrifugation at $560g_{av}$ for 10 minutes, in Krebs-Henseleit buffer containing 0.5%(w/v) bovine serum albumin. The cells were resuspended in this buffer and incubated at room temperature for 1 hour. Cell viability was determined with trypan blue (0.006% w/v), and neutral red (0.003% w/v) was used to determine the percentage of chromaffin cells.

For cell-labelling experiments, the cells were suspended in RPMI-1640 (Gibco) in the absence of methionine. Cells were incubated in this medium for 30 minutes at $37^{o}C$ prior to the addition of ^{35}S-methionine. Between 25-50uCi ^{35}S-methionine were added per 10^{6} cells. For pulse-chase experiments, cold methionine was added to 10mM. Approximately $1.5x10^{6}$ cells were loaded on each isoelectric focusing gel.

2.03.: METHODS FOR TRANSLATION SYSTEMS

2.03.01.: Preparation of Rabbit Reticulocyte Lysate

Rabbit reticulocyte lysate was prepared essentially as described by Hunt and Jackson, 1974.

A New Zealand white female rabbit, weighing about 2.5kg was injected subcutaneously in the scruff of the neck, on 5 consecutive days with 1ml of filtered (pore size 0.45µm), neutralized phenylhydrazine solution (0.23M phenylhydrazine, 2.5mM glutathione

(reduced), adjusted to pH7 with NaOH). This treatment destroys red blood cells, thus inducing erythropoiesis. The rabbit's blood cells were examined daily by smearing one drop of blood onto a microscope slide. This was air dried before adding 10 drops of Leishman's stain, followed 1 minute later by 20 drops of water. After 15 minutes, the slide was washed with water and air dried. Leishman's stain stains erythrocytes yellow and reticulocytes blue.

The rabbit was sacrificed on day 7 or day 8 after the first injection, when the balance between the percentage of reticulocytes present and the haematocrit was optimal. Prior to exsanguination, the rabbit was injected intravenously with 1ml of normal saline (0.13M NaCl, 7.5mM $MgCl_2$, 5mM KCl) containing 200 units of heparin, followed by 5ml of Nembutal. The blood was collected, by cardiac puncture, into a beaker containing 25ml ice-cold normal saline and 100 units of heparin. Subsequent procedures were performed at $4^{o}C$.

The blood was centrifuged at $1,500g_{av}$ (5,000 rpm) for 15 minutes in a Beckman JA-20 rotor. The plasma was discarded and the blood cells were gently resuspended in a similar volume of normal saline and centrifuged as before. This procedure was repeated 5 times in order to remove the buffy coat and heparin. After the final wash, the blood cells were lysed by the addition of one volume (equal to the volume of packed cells) of triple glass-distilled, sterile water containing 0.5mM dithiothrietol, which preserves endogenous ribonuclease inhibitor. The suspension was gently swirled for 5 minutes and then centrifuged at $30,000g_{av}$ (15,000 rpm)in a Beckman JA-20 rotor for 15 minutes. The supernatant was aliquoted (1ml) into

sterile, EDTA-treated Eppendorf tubes, quick frozen in liquid nitrogen and stored at -140°C.

2.03.02.: The Thawing of the Reticulocyte Lysate and Translation of the Endogenous Reticulocyte messenger RNA

A 1mM solution of haemin in 90% (v/v) ethylene glycol, 50mM Tris-HCl, pH8 (the haemin was first dissolved in 1/50 final volume of 0.05M KOH) was added to the lysate to a final concentration of 25mM as it began to thaw. To translate the endogenous reticulocyte messenger RNA, creatine kinase (5mg/ml in 50% aqueous glycerol) was added to the lysate to 50µg/ml and used directly for translation. To 30µl of reticulocyte lysate was added 4µl of translation cocktail*, 2.6µl of 4mM EDTA, 4µl 1M KCl, 4µl (20µCi) ^{35}S-methionine and water to 52µl. The optimal concentrations of EDTA and K^+ were obtained as described in Section 4.02.02 and 4.02.03.. The translation reactions were incubated at 30°C for 60 minutes.

* The translation cocktail, according to Maniatus et al., 1982, consisted of 6.25mM spermidine, 100mM creatine phosphate, 25mM dithiothreitol, 250mM HEPES-NaOH, pH7.4, and 300µM each of 19 L-amino acids. Aliquots were stored at -70°C.

2.03.03.: Preparation of Message-Dependent Reticulocyte Lysate

Endogenous reticulocyte messenger RNA was destroyed by treating the lysate with Ca^{2+}-activated micrococcal nuclease, as described by

Pelham and Jackson, 1976. After the addition of haemin to the lysate to a concentration of 25μM, $CaCl_2$ was added to the thawed lysate to a final concentration of 1mM. Micrococcal nuclease was then added to a concentration of 5μg/ml. The lysate was gently mixed and then incubated for 15 minutes at 20°C. The micrococcal nuclease was subsequently inactivated by the addition of EGTA (pH7) to 2mM. Creatine kinase was added to 50μg/ml and 150μl aliquots were quick frozen in liquid nitrogen and stored at -70°C. Aliquots, once thawed, were not refrozen.

2.03.04.: Translation of Messenger RNA in Message-Dependent Reticulocyte Lysate

All solutions were kept on ice. To a sterile Eppendorf tube was added 4μl of 1M KCl, 4μl translation cocktail*, 2.6μl 4mM EDTA (pH7), and 20-30μCi ^{35}S-methionine. The volume was adjusted to 19.4μl by the addition of water. Immediately after thawing, 30μl of message-dependent reticulocyte lysate was added. Finally, 2.6μl of adrenal medullary poly A^+ RNA (1μg/ul), which had been heated at 65°C for 10 minutes and then cooled on ice, was added. The translation mix was incubated at 30°C for 60 minutes. Translation was halted by transferring the reaction mix to ice. The volume of the translation reaction mix was scaled up or down as appropriate. Optimal concentrations of KCl, EDTA and poly A^+ RNA were determined as described in Sections 4.03.01. and 4.03.02..

2.03.05.: Determination of the Percentage of ^{35}S-Methionine Incorporated into TCA-Precipitable Material

The method recommended by Hunt and Jackson, 1974, was used. Samples (1µl) were removed from the translation reactions into 0.5ml of 1M NaOH containing 5%(v/v) H_2O_2. After incubation for 10 minutes at 37°C to hydrolyse aminoacyl-tRNA complexes and to bleach haemoglobin, 3ml of ice-cold 25% trichloracetic acid, containing 2% casein acid hydrolysate was added. The solutions were left on ice for at least one hour. Precipitated material was collected on nitrocellulose discs (pore size 0.45µm) and washed with two 5ml volumes of ice-cold 8% trichloracetic acid. Filters were dried and counted in 2.5ml of toluene fluor at an efficiency of 90-95% in a Searle Mark III scintillation counter. Duplicate samples were routinely processed, and a 1µl sample from the translation reaction was spotted directly onto a filter disc and counted to determine the amount of ^{35}S-methionine present per µl of translation mix.

2.03.06.: Preparation of Dog Pancreas Microsomes

The procedure used was essentially that of Walter and Blobel, 1983b. The dog pancreas, usually weighing about 50g, was excised immediately after death and rinsed with ice-cold buffer A (250mM sucrose, 50mM Tris-HCL, pH7.4, 25mM KCl, 5mM $MgCl_2$, 2mM dithiothreitol, 1mM EDTA). All subsequent steps were performed at 4°C. After removing fat and major blood vessels, the pancreas was chopped with scissors, and passed through a stainless steel mincer. The pancreas was homogenised with 5 strokes in a Potter-Elvehjem

glass homogeniser with a loose-fitting teflon pestle in buffer A (4ml buffer/g tissue). Cell debris was removed by centrifugation at $1,500g_{av}$ (3,000rpm) for 10 minutes in a Beckman JA-20 rotor. Floating fatty material was removed by aspiration and the supernatant was centrifuged at $14,000g_{av}$ (9,500 rpm) for 10 minutes in a Beckman JA-20 rotor. The resulting supernatant was carefully decanted and crude rough microsomes were collected by centrifugation through a discontinuous sucrose gradient in 13.5ml polyallomer centrifuge tubes containing 1.5ml 1.3M sucrose and 3ml 2.25M sucrose, both in buffer A. The sucrose gradients were centrifuged at $196,000g_{av}$ (40,000rpm) for 60 minutes in a Beckman SW41 rotor. Rough microsomes were collected from the 1.3M/2.25M sucrose interface and pooled. Aliquots of 100µl were quick frozen in liquid nitrogen and stored at $-70^{o}C$. Once thawed, the microsomes were not refrozen. The absorbance of the microsomes at 260nm and 280nm was determined in 0.2%(w/v) SDS, and the $A_{260}:A_{280}$ ratio calculated. The ratio was always about 1.9.

2.03.07.: Preparation of Dog Pancreas Microsomes for use in <u>in vitro</u> Processing Reactions

The endogenous dog pancreas microsome bound polysomes may be destroyed by EDTA extraction or by micrococcal nuclease treatment. For use in the reticulocyte lysate system, micrococcal nuclease-treated dog pancreas microsomal membranes are superior to EDTA-stripped membranes (Scheele <u>et al</u>., 1980).

The aliquot of dog pancreas microsomes was rapidly thawed by

agitation. $CaCl_2$ was added to the microsomes to a final concentration of 1mM, followed by the addition of micrococcal nuclease to 30μg/ml. The mix was incubated at 20^{o}C for 30 minutes. The micrococcal nuclease was then inactivated by the addition of EGTA to a final concentration of 2mM. The microsomal membranes were cooled on ice and used immediately in translation reactions at a concentration of 5 A_{260} units/ml (Section 4.06.01.). Microsomes were added to the translation reaction after the addition of reticulocyte lysate but immediately before the addition of the poly A^+ RNA.

2.03.08.: Isolation of Microsomal Vesicles after the Translation Reaction

After the translation reaction, microsomal vesicles were recovered by centrifugation. Screw-cap 1.5ml Eppendorf tubes were centrifuged at 35,000g_{av} (20,000rpm) in a Beckman 70.1Ti rotor for 30 minutes. Rotor wells containing Eppendorf tubes had 5ml of water added to them to support the tube walls. After centrifugation, the supernatant was removed leaving a barely-visible pellet of microsomal vesicles. The vesicles were washed by resuspending them in 0.15M sucrose, 10mM HEPES-NaOH, pH7, 1mM PMSF and recentrifugation.

2.03.09.: Post-Translational Proteolysis Assay

This assay for functional dog pancreas microsomes was performed essentially as described Morimoto by et al., 1983). Translations in

the presence of nuclease-treated dog pancreas microsomes were stopped after incubation for 60 minutes at 30°C by the addition of cycloheximide to 10µg/ml reaction. Tetracaine in 77mM KCl, 3mM $MgCl_2$ was added to a final concentration of 3mM in order to stabilize the membranes by preventing the activation of a phospholipase, thereby improving the effectiveness of the microsomes to protect against proteases (Scheele et al., 1980). The mix was incubated at 20°C for 5 minutes and then placed on ice. $CaCl_2$ was added to 8mM, either to activate pronase or to prevent the autolysis of trypsin. The mix was then incubated for 90 minutes on ice in the presence of either 3µg each of chymotrypsin and trypsin or 3µg of pronase. Proteolysis was stopped by the addition of soya bean trypsin inhibitor and PMSF to 5mM to inhibit trypsin and chymotrypsin respectively or EGTA to 20mM to inhibit pronase. The appropriate gel sample buffer was then added. The control proteolysis experiment was made 1%(v/v) in Triton X-100 before the addition of the proteases.

2.03.10.: The Separation of Microsomal Vesicle Membranes from Microsomal Vesicle Contents using Dilute Alkali Solution

The contents of the microsomal vesicles were removed by the method of Fujika et al., 1982. The microsomal vesicles from a translation reaction were isolated and washed by centrifugation as described in Section 2.03.07.. The microsomal vesicles were lysed and the contents removed by resuspending the microsomal membrane pellet in 100mM Na_2CO_3, 1mM EDTA, 1mM PMSF followed by incubation on ice for 30 minutes. The microsomal membranes were pelleted by

centrifugation as before. The supernatant, containing the microsomal contents, was dialysed against 1mM HEPES-NaOH, pH7, 0.1mM EDTA, 1mM PMSF, and then lyophilized and resuspended in the appropriate gel sample buffer. The membrane pellet was washed with 10mM HEPES-NaOH, pH7, 1mM EDTA, 1mM PMSF, recentrifuged, and suspended in the appropriate gel sample buffer.

2.03.11.: Separation of Microsomal Membranes from Microsomal Contents using Triton X-114

The method of Bordier (1981) as described by Pryde and Phillips, (1985), was used to separate microsomal membranes from microsomal contents. The dog pancreas microsomes, isolated from a translation reaction by centrifugation as described in 2.03.07., were resuspended in 80µl Tris-HCl, pH7.6 and 20µl of 10% Triton X-114 was added. After incubation for 5 minutes on ice, the mix was centrifuged as described above. The resulting supernatant was layered over 30µl of 0.25M sucrose and incubated at 30°C for 5 minutes. The tube was then centrifuged for 5 minutes in an MSE microfuge at $12,800g_{av}$ and the supernatant removed, leaving behind most of the sucrose cushion. The supernatant was made 0.5% Triton X-114, incubated on ice for 5 minutes, then layered over the same sucrose cushion as before and incubated at 30°C for 5 minutes. The mix was then centrifuged as before in an MSE microfuge and the supernatant removed, leaving behind the detergent-rich pellet. The supernatant was made 2%(v/v) in Triton X-114, incubated on ice for 5 minutes, centrifuged in an MSE microfuge and the supernatant, containing the soluble contents, carefully removed. The walls of the

Eppendorf tube containing the detergent-rich pellet were washed with Tris-HCl, pH7.6 and then microfuged for 5 minutes. This procedure was repeated before removing the sucrose cushion. To the pellet was added 100µl of Tris-HCl, pH7.6, and this was incubated at 30°C for 5 minutes before microfuging for 5 minutes. The supernatant was discarded, the sucrose cushion was washed with Tris-HCl, pH7.6 and the sucrose cushion was removed. About two volumes of Tris-HCl, pH7.6, was added to the viscous, detergent-rich pellet, containing the microsomal membrane proteins.

2.03.12.: Immunoprecipitation

The procedure used was that of Anderson and Blobel, 1983. The translation mix or appropriate fraction was made 4%(w/v) SDS and heated to 96°C for 5 minutes. One volume of water was added, reducing the SDS concentration to 2%(w/v), followed by the addition of four volumes of ice-cold Triton buffer (2.5%(w/v) Triton X-100, 190mM NaCl, 60mM Tris-HCl, pH7.4, 6mM EDTA, 1mM PMSF, 1mM Benzamidine). Between 5 and 25µl of the appropriate antiserum was added and the sample was incubated on ice for 20-24 hours.

Inactivated S. aureus cells were washed three times with a buffer containing 2%(w/v) Triton X-100, 0.4%(w/v) SDS, 150mM NaCl, 50mM Tris-HCl, pH7.4, 5mM EDTA, 1mM PMSF, 1mM benzamidine. The cells were pelleted each time by centrifugation for 10 minutes at 1,200g_{av} (3,200rpm) in a MSE benchtop centrifuge at room temperature, and finally resuspended to 50%(v/v) in the 2%(w/v) Triton buffer. Washed

S. aureus cells (100µl) were added to the immunoprecipitation, and the mixes were rotated for 2-4 hours at room temperature. The cells were then pelleted by centrifugation for 2 minutes in an MSE microfuge at the "slow" setting. The supernatant was discarded and the cells were washed 5 times by centrifugation and resuspension in a wash buffer (0.1%(w/v) Triton X-100, 0.02%(w/v) SDS, 150mM NaCl, 50mM Tris-HCl pH7.4, 5mM EDTA, 1mM PMSF, 1mM benzamidine). If the sample was be subjected to 2-dimensional gel electrophoresis, a final wash with buffer without detergents was included. The antibody-antigen complexes were eluted from the cells by resuspension in about 50µl of the appropriate gel sample buffer (if 2-dimensional gel sample buffer was used, ampholines were not included) and the sample was boiled for 5 minutes. Free -SH groups were blocked by adding iodoacetamide to 0.2M, followed by incubation for 45 minutes at 37°C. The S. aureus cells were pelleted by centrifugation for 5 minutes in an MSE microfuge at $11,800g_{av}$. The supernatant was withdrawn and stored on ice prior to electrophoresis. Ampholines were added to samples which were to be subjected to isoelectric focusing.

2.03.13.: Preparation of Wheatgerm Extract

The method used was essentially that of Roberts and Paterson, 1973, as modified by Dr C. Leaver, Botany Department, University of Edinburgh. The whole procedure was performed at 4°C. Five grams of Wheatgerm was ground with 5g of glass beads in 15ml of Grinding Buffer (50mM HEPES-NaOH, pH7.6, 5mM $Mg(acetate)_2$, 120mM Kacetate, 1.5mM dithiothreitol). The suspension was centrifuged for 15 minutes at $30,000g_{av}$ (15,000rpm) in a Beckman JA-20 rotor. The supernatant

was removed and applied to a G25 Sephadex column which had been equilibrated with Grinding buffer. Fractions of 1ml were collected and samples of the fractions were diluted 1/100 and their A_{260} determined. The most concentrated fractions were pooled and centrifuged for 15 minutes at $30,000g_{av}$ (15,000rpm) in a Beckman JA-20 rotor. The resulting supernatant was dialysed against 10,000 volumes of Grinding buffer, aliquoted and quick frozen in liquid nitrogen for storage at $-70^{o}C$.

2.03.14.: Translation of Messenger RNA in the Wheatgerm S-30 System

Translations of messenger RNA were performed in wheatgerm extract as recommended by Dr. C. Leaver, Botany Department, University of Edinburgh.

Translation reactions were performed in a volume of 50ul and contained 20µl wheatgerm extract, 28mM HEPES, NaOH, pH7.6, 78mM KCl, 2.25mM $Mg(Acetate)_2$, 0.25mM spermidine, 1mM ATP, 50µM GTP, 8mM creatine phosphate, 5µg creatine kinase, 2mM dithiothreitol, 25µM of each amino acid, 20-30uCi ^{35}S-methionine and 2.5ug poly A^{+} RNA. The reaction was incubated at $25^{o}C$ for 60 minutes. The percentage of ^{35}S-methionine incorporated into TCA-precipitable material was determined as described in Section 2.03.05..

2.03.15.:Preparation of Rat Liver High Speed Supernatant

(from Ramsey and Steele, 1976)

Six rat livers (total weight about 50g) were washed in ice-cold 0.25M sucrose, 3mM dithiothreitol. The livers were chopped with scissors, passed through a stainless steel mincer and resuspended in 100ml of 0.25M sucrose, 3mM dithiothreitol. The suspension was homogenised with 10 strokes in a glass Potter-Elvehjem homogeniser with a loose-fitting teflon pestle. The homogenate was centrifuged for 20 minutes at $30,000g_{av}$ (15,000rpm) in a Beckman JA-20 rotor at $4^{o}C$. The resulting supernatant was centrifuged for 90 minutes at $227,000g_{av}$ (50,000rpm) in a Beckman 50.2Ti rotor at $4^{o}C$. Some of the resulting supernatant was retained for fractionation on a Biogel P-100 column (Section 2.03.16). The remainder was aliquoted and quick frozen in liquid nitrogen for storage at $-70^{o}C$.

2.03.16.: Preparation of a P-100 Fraction Derived from Rat Liver High Speed Supernatant

A 30cm x $15.9cm^2$ column, packed with Biogel P-100 and equilibrated with 50mM Tris-HCl, pH7.4, 50mM KCl, 5mM $MgCl_2$, was used. About 15ml of rat liver high speed supernatant was applied to the column and eluted with the same buffer. The flow rate was 30ml/hour. Fractions of 5ml were collected, and their A_{280} determined. The most concentrated fractions were pooled and aliquots were quick frozen in liquid nitrogen and stored at $-70^{o}C$. The final protein concentration of the P-100 fraction was usually around

10mg/ml and the A_{260}:A_{280} was usually around 0.7.

2.04.: Gel Electrophoresis and Related Techniques

2.04.01.: SDS-Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was performed according to Laemmli and Favre, 1973. Exponential gradient gels of 8% to 15%(w/v) polyacrylamide were routinely used. However, straight 10% and 15% polyacrylamide gels were prepared similarly.

Glass gel plates (16cmx20cm) were thoroughly cleaned with ethanol and the gel cassette assembled with 3x1mm thick spacers. The gel cassette was secured with bulldog clips and sealed with 1.5%(w/v) hot agar.

The gel solutions required were as follows:-

A. 4X Separating Gel Buffer: 1.5M Tris-HCl, pH8.8 (@20^{o}C), 8mM EDTA (disodium salt), 0.4%(w/v) SDS

B. Acrylamide solution: 30%(w/v) acrylamide, 0.8%(w/v) N,N', methylene bisacrylamide

C. 6X Polyacrylamide solution: 3%(w/v) polyacrylamide

D. 4X Stacking Gel Buffer: 0.5M Tris-HCl, pH6.5 (@20^{o}C), 4mM EDTA, 0.4%(w/v) SDS

E. 4X Sample Buffer: 0.5M Tris-HCl, pH6.5 (@20^{o}C), 8%(w/v) SDS, 0.2M EDTA (pH7), 40%(v/v) glycerol

F. 5X Electrode Buffer: 0.25M Tris-HCl, pH8.3, 1.9M Glycine, 0.01M

EDTA, 0.5%(w/v) SDS.

The gel cassette held 30ml of separating gel. Two solutions were prepared, 10ml of the highest concentration of acrylamide (e.g.15%) and 20ml of the lowest concentration of acrylamide (e.g.8%), both containing 1X separating gel buffer and 0.5%(w/v) polyacrylamide. Freshly-prepared 10%(w/v) ammonium persulphate and TEMED were added to each solution to a concentration of 0.06%(w/v) and 0.05%(v/v) respectively. Using an Gilson pump, the 8% gel acrylamide solution was delivered to and mixed with the 15% acrylamide gel solution. Simultaneously, the originally 15% acrylamide gel solution was delivered to the assembled gel cassette. The gel was poured to within 2-3cm from the top of the gel plate, overlaid with water-saturated butan-2-ol, and allowed to polymerise. After polymerisation (about 20 minutes), the butan-2-ol was washed from the gel surface with distilled water. The stacking gel buffer containing 4.5%(w/v) acrylamide, 1X stacking gel buffer, 0.5%(w/v) polyacrylamide, 0.1%(v/v) TEMED and 0.1%(w/v) ammonium persulphate, was poured on top of the separating gel and a gel comb, to produce the desired number of sample wells, was inserted between the plates. After polymerisation of the stacking gel (5-10 minutes), the gel comb and the bottom spacer were removed from the gel cassette. The cassette was cleaned, greased, and clipped onto a gel tank. Electrode buffer (1X concentrated) was added to the top and bottom tank chambers. Sample wells were cleaned of non-polymerised acrylamide and trapped air was expelled from the bottom of the gel using a bent syringe needle. Samples were loaded onto the gel wells and electrophoresis was performed overnight for about 16 hours at

60-65 Volts.

Samples were prepared in 1X concentrated sample buffer in volumes of 50-100µl, depending on the size of the gel wells. If reducing conditions were desired, 2-mercaptoethanol was added to 1%(v/v). Bromophenol blue was added to 0.001%(w/v). Samples were delipidated if necessary, by precipitation at 0°C for 30 minutes in acetone:ethanol 1:1 v/v. Proteins were pelleted by centrifugation at $75,000g_{av}$ (30,000rpm) in a Beckman SW50 rotor for 30 minutes at 4°C.

After electrophoresis, the gel cassette was disassembled and the polyacrylamide gel removed and placed in about 200ml of Gel Fix (10%(v/v) acetic acid, 20%(v/v) methanol) for at least 30 minutes. The gel could then be stained by incubation for 20 minutes in 0.25%(w/v) Coomassie Brilliant Blue R250, dissolved in 50%(v/v) methanol, 7.5%(v/v) acetic acid. Gels were destained in 10%(v/v) methanol, 7%(v/v) acetic acid. Destaining was accelerated by including a piece of polyurethane packing foam and/or by incubation at higher temperatures (40-50°C). After destaining, the gel was dried under vacuum on a gel drier for 15 minutes at 80°C followed by a further 25 minutes while cooling. When ^{35}S-labelled proteins were to be detected, gels were salicylated before being dried down, as described in Section 2.04.02.

2.04.02.: Autoradiography and Fluorography

^{125}I-labelled proteins were detected by autoradiography. In total darkness, dried gels were placed in cassettes and a sheet of

Agfa-Gevaert Curix RP-1 X-ray film placed on top. The films were exposed for 1-2 days at -70°C. ^{35}S- and ^{3}H-labelled proteins were detected by fluorography as described by Chamberlain, 1979. After fixing or destaining, gels were washed for 5-10 minutes in water and then shaken for 30-45 minutes at room temperature in 250ml of freshly prepared 1M sodium salicylate. Gels were dried under vacuum and exposed normally for 4 days, at -70°C.

2.04.03.: Cleveland Gels

Gels were prepared and run as described by Cleveland et al., 1977.

Protein bands were excised from a stained gel (Section 2.04.01), soaked in water and then for 15 minutes in 1X concentrated sample buffer (Section 2.04.01.). A 2mm thick, 15%(v/v) polyacrylamide gel with a 4.5cm stacking gel was prepared. Gel pieces were placed in each gel well and the wells were filled with 1X stacking gel buffer before filling the gel tank chambers with 1X electrode buffer. About 25µl of sample buffer containing 20%(v/v) glycerol was added to each well. 5µl of varying concentrations (0.5mg/ml, 0.05mg/ml, etc. down to 0.000005mg/ml) of the appropriate protease were added very carefully in 10µl of sample buffer containing 10%(v/v) glycerol to the gel wells. Electrophoresis was performed slowly until the samples entered the separating gel. Mercaptoethanol and EDTA were omitted from the sample buffers. Gels were treated as usual after electrophoresis except that prior to drying, they were soaked in 15%(v/v) glycerol to prevent cracking.

2.04.04.: Isoelectric Focusing

Isoelectric focusing was performed as described by O'Farrell et al., 1975. Chromic acid-washed, hollow, 14cm glass tubes with an internal diameter of 2.5mm were used. They were sealed at the bottom with parafilm and a marked, 11.5cm from the bottom. The following solutions were required:-

A. 45%(w/v) acrylamide, 0.6%(w/v) N,N', methylene bisacrylamide

B. 10%(w/v) Triton X-100

C. Ampholines 40%(w/v)

D. 0.4%(v/v) ethanolamine

E. 0.2%(v/v) phosphoric acid

F. Sample Buffer containing 9.5M urea, 1%(w/v) Triton X-100, 1%(w/v) ampholines, 0.4%(v/v) 2-mercaptoethanol and bromophenol blue to 0.001%(v/v).

G. Overlay solution containing 5M Urea, 0.4%(w/v) ampholines.

The appropriate amount of gel solution (9.5M urea, 4.5%(w/v) acrylamide, 0.06%(w/v) bisacrylmide, 2%(w/v) Triton X-100, 1.61%(w/v) ampholine) was prepared and degassed. Freshly prepared ammonium persulphate was added to 0.04%(w/v) and the gel solution was poured immediately into the glass tubes up to the 11.5cm mark. The gels were overlaid with solution G and allowed to polymerise. After polymerisation, the overlay solution was shaken from the tube gels and they were mounted on the tube gel tank. The tank containing the acid solution, usually the bottom tank for better resolution of acidic proteins, served as the anode. Lyophilized samples containing

about 300µg protein were resuspended in 2-dimensional gel sample buffer. Alternatively, at least 2 volumes of sample buffer were added to the sample. A maximum volume of 100µl was loaded. The samples in sample buffer were loaded onto the tube gels and overlaid with 10µl of solution G. The top tank buffer was then carefully added. Electrophoresis was begun at 100V for 10 minutes, then increased to 200V for 30-60 minutes and finally increased to 400V and left for about 20 hours at room temperature. Thirty minutes before taking the gels off, the voltage was increased to 800V. Tube gels were removed from the tubes and soaked in soaking buffer (3%(w/v) SDS, 3%(v/v) 2-mercaptoethanol, 50mM Tris-HCl, pH6.8, 10%(v/v) glycerol and 0.005%(w/v) bromophenol blue) for 30-60 minutes. Meanwhile, the second dimension SDS-polyacrylamide gels were prepared as described in Section 2.04.01., but with a 1cm stacking gel. Each tube gel was secured to the top of a separating gel attached to a gel tank with a hot 1%(w/v) agarose solution in soaking buffer. Second dimension gels were treated as normal one-dimensional SDS-polyacrylamide gels, except that they were fixed for at least 2 hours to remove the ampholines before staining.

2.04.05.: Western Blotting and Immune Replicas

The method used was that of Towbin et al., 1979. Proteins were electrophoretically transferred from SDS-polyacrylamide gels to nitrocellulose sheets. The following solutions were required:-

A. Electroblot buffer: 25mM Tris-HCl, pH8.6, 0.19M Glycine, 20%(v/v) methanol

B. Blocking Buffer: 20mM Tris-HCl, pH7.2, 0.15M NaCl, 30mg/ml BSA

and 0.5mg/ml sodium azide

C. Horse serum Buffer: 20mM Tris-HCl, pH7.2, 0.15M NaCl, 5%(v/v) heat-inactivated horse serum and 0.5mg/ml sodium azide

D. 20mM Tris-HCl, pH7.2, 0.15M NaCl

Immediately after electrophoresis, the gel was removed from the gel cassette and placed on top of Whatman 3MM fiter paper which had been soaked in electroblot buffer. Nitrocellulose filter paper (pore size 0.45μm) was soaked in electroblot buffer and placed on top of the gel, taking care to avoid trapping air bubbles between the gel and the nitrocellulose. Another sheet of soaked 3MM paper was laid on top of the nitrocellulose. These were then sandwiched between Scotch-Brite scouring pads and placed on the electroblot tank grid which was then sealed tightly with 2 elastic bands. Transfer was for 1-2 hours at 6V/cm in electroblot buffer. After transfer, the nitrocellulose paper was removed and incubated for 2 hours at room temperature in blocking buffer. Following this, the nitrocellulose was incubated for about 60-90 minutes with antiserum, diluted 50 times with horse serum buffer and then washed 5 times for 5-10 minutes with of Tris-salt buffer. Triton X-100 was included in the third wash to a concentration of 0.5% (w/v). ^{125}I-labelled protein A (about 0.5μCi/ml) in Tris-salt buffer was then used to decorate the antibodies on the nitrocellulose paper. This 60 minute incubation was followed by 5 washes with Tris-salt buffer as before. The nitrocellulose sheet was air dried and autoradiographed for about 2-4 days at room temperature.

2.04.06.: Electroelution of Proteins from SDS-Polyacrylamide Gels

The appropriate stained gel slice was excised from a gel, soaked in water and then in Electrode buffer (Section 2.04.01.). Biorad electroelution cassettes were placed in a flat-bed gel tank with 1X electrode buffer just covering the bottom of each chamber. The gel slices were placed in the large chamber and electrode buffer in the small chamber, with sufficient to cover the bridge between the chambers. Electroelution was performed for about 16 hours at 40V. Electroeluted protein was collected from the small chamber and dialysed against 10%(v/v) ethanol (3 changes; 100 volumes). The protein was lyophilized and resuspended in the desired buffer.

2.04.07.: Electrophoresis of RNA through Agarose Gels containing Formaldehyde

The method used was that described by Maniatus et al., 1982. The solutions required were:-

A. 5X gel running buffer: 0.2M morpholinopropanesulphonic acid (MOPS), pH7, 50mM sodium acetate, 5mM EDTA

B. Loading buffer: 50%(v/v) glycerol, 1mM EDTA, 0.4%(w/v) bromophenol blue

C. Sample: 10-20μg RNA in 1X gel running buffer, 6.5%(w/v) formaldehyde, 10%(v/v) deionised formamide, incubated at 55°C for 15 minutes.

A 1%(w/v) agarose solution was prepared in 1X gel running buffer containing 2.2M formaldehyde, 1μg/ml ethidium bromide, and

poured and allowed to set on a 4cmx6cm glass plate. The gel was placed in a flat-bed gel electrophoresis tank and 1X running buffer, containing 0.1μg/ml ethidium bromide, was added until the surface of the gel was covered. To the RNA sample was added 0.1 volume of loading buffer. The sample was loaded into the gel well and electrophoresis was performed for 3-4 hours at 30V. The RNA was visualized under ultraviolet light.

2.05.: Other Procedures

2.05.01.: Production of Antibodies

Young, New Zealand white female rabbits were used. For the primary injection, 0.5ml of protein (1mg/ml) was mixed with an equal volume of Freunds complete adjuvant (Difco) in a Dupont omni-mixer. The emulsion was injected intramuscularly. Six weeks later, a secondary injection of 0.25ml (1mg/ml) protein mixed with an equal volume of Freunds incomplete adjuvant was injected. Ten to fourteen days later, 50ml of blood was collected from the ear vein of the rabbit. The blood was allowed to clot overnight at $4^{o}C$, and the serum was collected and centrifuged at $2,000g_{av}$ (5,000rpm) in a Beckman JA-20 rotor for 10 minutes at $4^{o}C$. Before use, the antiserum was heated at $55^{o}C$ for 30 minutes, then filtered (pore size 0.45μm). The antiserum was stored in small aliquots at $-70^{o}C$.

2.05.02.: Preparation of Immunoglobulins G from Antiserum

A column of 30cm x $2.25cm^{2}$ was packed with DEAE Affi-blue gel

and equilibrated with Running Buffer (20mM Tris-HCl, pH8@4^{o}C, 0.028M NaCl). About 8ml of antiserum which had been dialysed against Running Buffer (3 changes; 100 volumes), was loaded onto the column. About 250ml of Running Buffer was used to elute the immunoglobulin G fraction. Fractions of 5ml were collected. The fractions of highest purity, as assessed by SDS-polyacrylamide gel electrophoresis, were pooled and the proteins precipitated overnight at 4^{o}C by the addition of 1 volume of saturated ammonium sulphate (i.e. 1.95M ammonium sulphate). Protein was collected by centrifugation for 20 minutes at 55,000g_{av} (25,000rpm) in a Beckman 50.2Ti rotor. Protein was resuspended in Running Buffer and dialysed against the same buffer (3 changes; 100 volumes). After lyophilization, the protein was stored at -70^{o}C. About 250ml of Elution Buffer (20mM Tris-HCl, pH8@4^{o}C, 1.4M NaCl) was run through the column before washing with 500ml of Wash Buffer (20mM Tris-HCl, pH8@4^{o}C, 1.4M NaCl, 8M urea).

2.05.03.: Sucrose Gradient Analysis of RNA and Polysomes

RNA was centrifuged through linear 5-30%(w/v) sucrose gradients containing 10mM Tris-HCl, pH7.5, 1mM EDTA, 0.5%(w/v) SDS. About 300μg of affinity purified poly A^{+} RNA was suspended in 200μl of 10mM Tris, HCl, pH7.5, 1mM EDTA, 0.5%(w/v) SDS and heated for 10 minutes at 65^{o}C to disrupt aggregates. Gradients were centrifuged for 16 hours at 160,000g_{av} (38,000rpm) in a Beckman SW41 rotor at 20^{o}C. Fractions of 300ul were collected and the RNA precipitated 3 times (Section 2.02.10) before use in translations.

Polysomes were centrifuged through linear 20-60%(w/v) sucrose

gradients containing 50mM Tris-Hcl, pH7.5, 50mM KCl, 5mM $MgCl_2$. Polysomes (about $12A_{260}$) were made 1%(w/v) in Triton X-100 and 0.5%(w/v) deoxycholate. The gradients were centrifuged for 4 hours at $196,000g_{av}$ (40,000rpm) in a Beckman SW41 rotor at $4^{o}C$.

2.05.04.: Elution of Radioactively-labelled Protein from SDS-Polacrylamide Gels

The method used was that according to Cabantchik and Rothstein, 1974.

Hydrated gel slices were finely chopped, either manually with a razor blade or with a gel slicer. The gel fragments were placed in 5ml scintillation vial inserts to which was added 0.3ml 15%(w/v) H_2O_2. The vials were incubated overnight at $65^{o}C$. Toluene-Triton scintillation fluor (3ml) was added to each vial, prior to counting in a Searle Mark III Scintillation counter. Elution was only 34% efficient as determined by eluting a known amount of radioactivity from a gel slice.

2.05.05.: Scintillation Fluors

Toluene Fluor:- To 2 litres of toluene was added 12.5g of 2,5,diphenyloxazole and 0.75g of 1,4,di-2-(5-phenyloxazolyl) benzene.

Toluene-Triton Fluor :- This fluor consisted of toluene fluor:Triton X-100 (scintillation grade) 2:1 (v/v)

2.05.06.: Protein Estimation by Dye Binding

Protein estimation was routinely performed using the Bradford assay (Bradford, 1976). Bradford reagent was made by dissolving 5mg of Serva Blue G in 5ml of 96% ethanol. To this was added 10ml of 85% orthophosphoric acid and the mix was stirred for 60 minutes at room temperature. The volume was adjusted to 100ml with water and the reagent filtered through glass wool. Bradford reagent was stored at $4^{o}C$. A standard curve was constructed using 1-10µg of bovine serum albumin. The concentration of bovine serum albumin was calculated from its absorption at 280nm. To each sample was added 1ml of Bradford reagent. After 30 minutes, but before 60 minutes, the samples were vortexed and their absorbance read at 595nm.

2.05.07.: Amino-terminal Amino Acid Determination using 4-NN-dimethylaminoazobenzene (DABITC)

The procedure described by Chang et al., 1978, and Chang, 1983, was followed. All glassware was washed in chromic acid and all solvents were redistilled as recommended by Chang, 1983. Briefly, the procedure involved dissolving 2nmol of lyophilized polypeptide in 40µl of 50%(v/v) pyridine to which was added 20µl of DABITC solution (10nmol DABITC in pyridine). This mix was incubated under nitrogen gas at $55^{o}C$ for 50 minutes before the addition of 5µl phenylisothiocyanate and a further incubation for 20 minutes at $55^{o}C$. Excess reagent was removed by three extractions with heptane-ethyl acetate 2:1 (v/v). The aqueous phase was dried under vacuum and dissolved in 50ul of 50% trifluoracetic acid and then incubated under

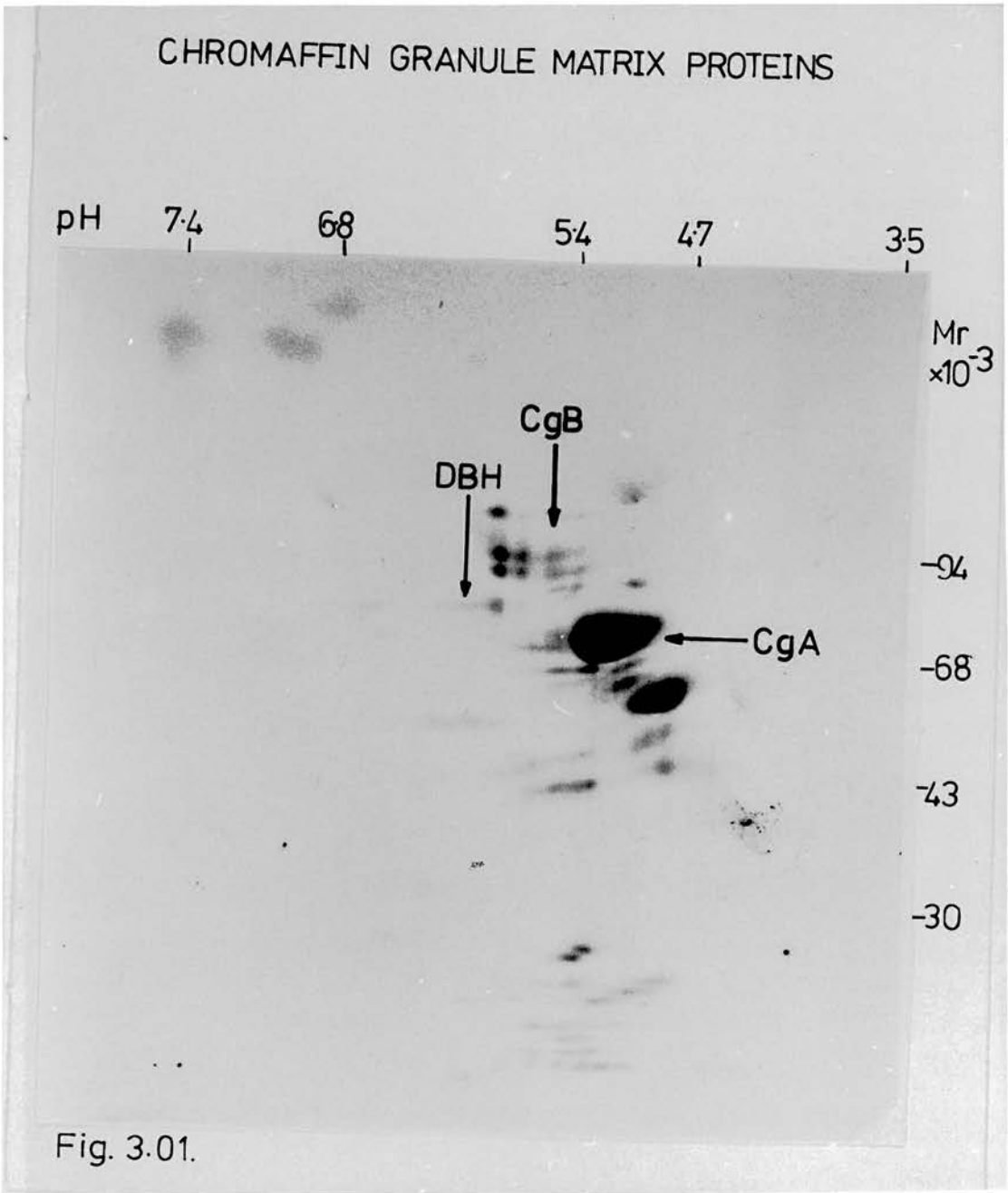
nitrogen gas for 10 minutes at 55°C. the trifluoracetic acid was evaporated and 50µl of water was added. The cleaved amino acid was extracted with butyl acetate and the butyl acetate was then evaporated. Finally, the extracted amino acid was dissolved in 50µl 50%(v/v) trifluoracetic acid and incubated under nitrogen gas for 45 minutes at 55°C. The acid was evaporated and the resulting dimethylaminoazobenzene thiohydantoin amino acid derivative was resuspended in 10µl of ethanol for analysis by thin layer chromatography.

Chromatography was performed in two dimensions on a $3cm^2$ polyamide sheet firstly with acetic acid:water 1:2 (v/v) and then with toluene:hexane:acetic acid 2:1:1 (v/v/v). The chromatograms were air dried and exposed to HCl vapour. Amino acids were identified by comparing their positions with amino acid standards, prepared as described by Chang, 1983. Leucine and isoleucine were resolved by 1-dimensional chromatography on silica gel plates with a solvent system of chloroform:ethanol 100:3 (v/v).

The system was tested by performing amino-terminal amino acid analysis on myoglobin, which has valine at its amino terminal.

CHAPTER 3

CHARACTERIZATION OF THE ANTISERA



Coomassie blue stained gel of chromaffin granule matrix proteins. 200ug of protein was loaded onto the isoelectric focussing gel.

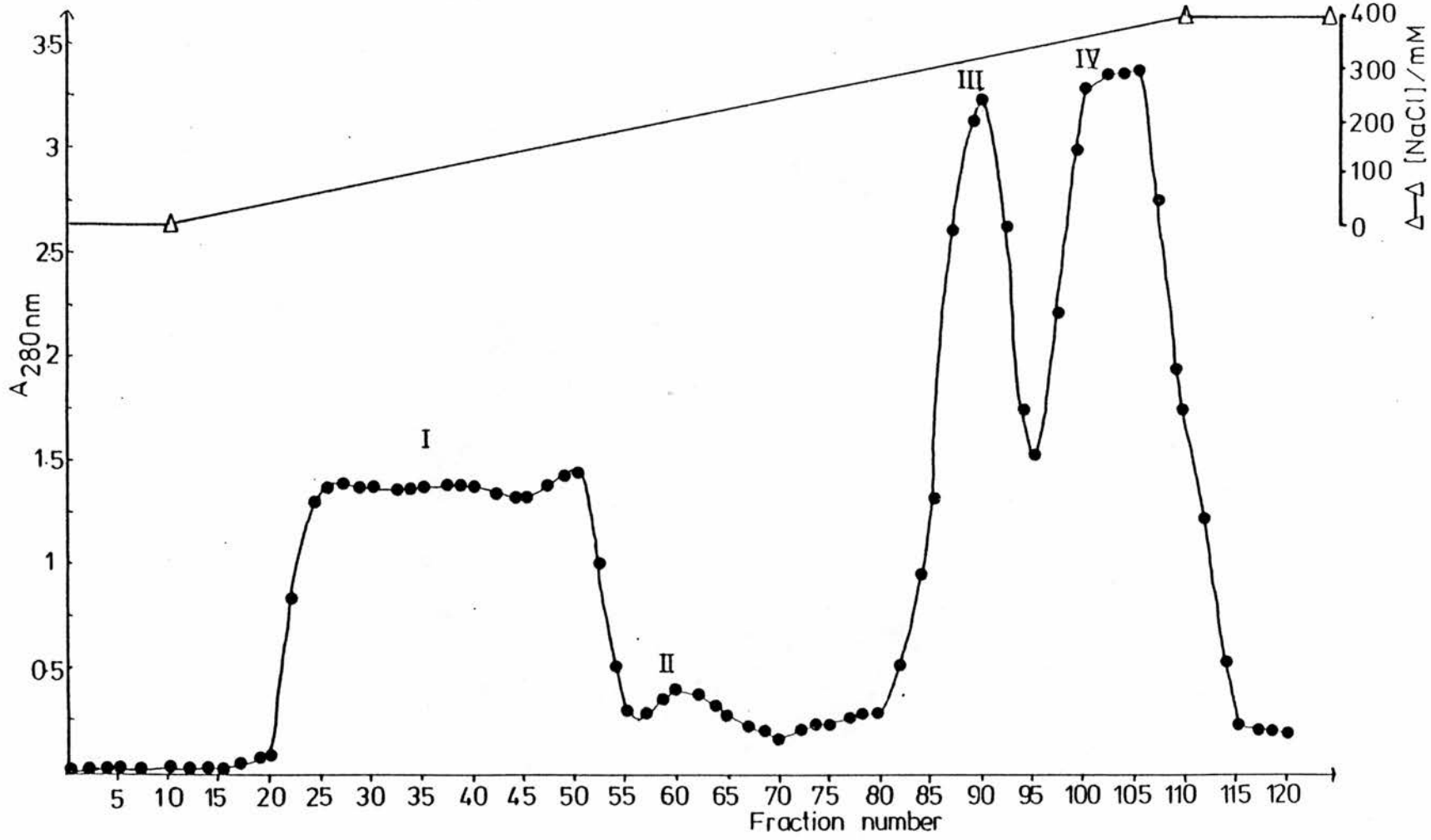
3.01.: Characterization of the Rabbit Antisera Raised against Chromogranin A, Soluble Dopamine β-Hydroxylase, and Cytochrome b_{561}

The antisera against chromogranin A, dopamine β-hydroxylase and cytochrome b_{561} were prepared by Drs. D.K. Apps and J.H. Phillips and had previously been characterized by the sensitive technique of immune-blotting (Section 2.04.05.). However, in this project these antisera were to be used to immunoprecipitate the precursors to the respective proteins against which they had been raised. It was therefore necessary first to characterize the antisera by their ability to immunoprecipitate the "mature" proteins from a mixture of ^{125}I-labelled proteins.

3.02.: Characterization of the Antisera Produced against Chromogranin A and Dopamine β-hydroxylase

In the past there has been much controversy over the separate identities of chromogranin A and soluble dopamine -hydroxylase (Winkler, 1976) and also, only recently has the chromogranin B family been identified as a group of chromaffin granule matrix proteins, similar, but immunologically distinct from the chromogranin A family (Falkensammer et al., 1985; Fischer-Colbrie and Frischenschlager, 1985). Examination of a 2-dimensional gel separation of the chromaffin granule matrix proteins (Fig.: 3.01.), makes obvious the reasons for the initial difficulties to characterize and purify chromogranin A and dopamine β-hydroxylase for the production of antisera to these proteins. The 75,000 dalton

Fig.: 3.02.:- Elution profile of chromaffin granule lysate proteins from DEAE cellulose column



subunit of dopamine β-hydroxylase is of similar molecular weight, but a little more basic than chromogranin A. Dopamine β-hydroxylase is a particularly good immunogen due to its carbohydrate composition and it must therefore, be completely removed from the chromogranin A preparation to be used for immunization. Chromogranin A was purified from chromaffin granule lysate (Section 2.02.02.) by DEAE-cellulose chromatography (Section 2.02.03.) which resolved the matrix proteins into 4 peaks as shown in Fig.3.02.. Peak I contained the most basic chromaffin granule matrix proteins, peak II contained most of the soluble dopamine β-hydroxylase, peak III contained the majority of the chromogranins A, and peak IV contained some chromogranins A and the chromogranins B. This chromatography step therefore resolved the chromogranin A family from the chromogranin B family, some of which co-migrate when separated only on a molecular weight basis by SDS-polyacrylamide gel electrophoresis. Initially however, this chromatography step was used as a precaution to prevent contamination of chromogranin A with dopamine β-hydroxylase. The proteins from peak III were then passed through a concanavalin A-sepharose column to remove any traces of mannose-containing glycoproteins, primarily dopamine β-hydroxylase. The resulting proteins from peak III, not absorbed by concanavalin A, were separated on a 10% SDS-polyacrylamide gel and the Coomassie blue-stained band of chromogranin A was excised and the protein electroeluted (Section 2.05.04.) from the gel. After dialysis against 10% ethanol (3 changes; 100 volumes) to remove as much residual SDS as possible, the purified chromogranin A was used to immunize a New Zealand white rabbit (Section 2.05.01.).

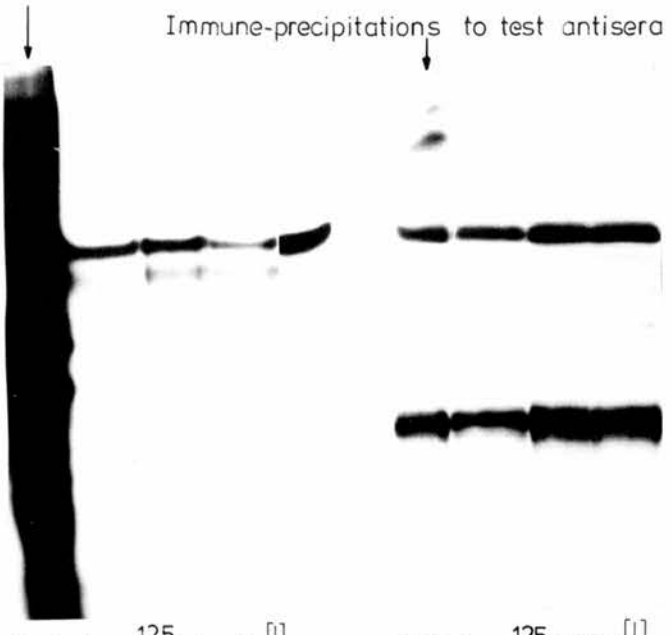
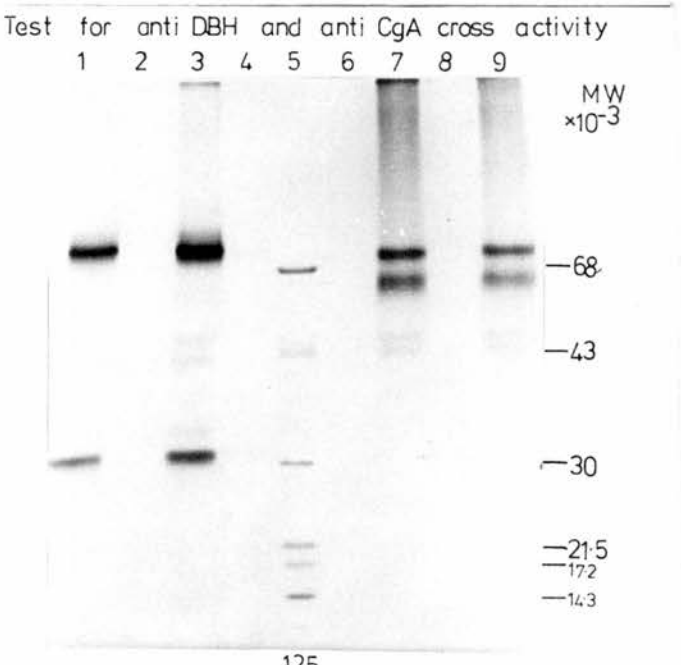


FIGURE:- 3.03



1. anti DBH against ^{125}I-DBH
2. anti CgA " "
3. anti DBH " " plus ^{125}I-lysate
4. preimmune serum against ^{125}I-DBH
5. MW standards
6. preimmune serum against ^{125}I lysate
7. anti CgA against ^{125}I-lysate plus ^{125}I-DBH
8. anti DBH " "
9. anti CgA " "

FIGURE:-3.04

Soluble dopamine β-hydroxylase was prepared from the peak II protein fraction obtained from DEAE-cellulose chromatography of chromaffin granule matrix proteins. The proteins from this fraction were passed through a concanavalin A sepharose column. Proteins not absorbed to the column were discarded. Absorbed proteins, the major component of which was dopamine β-hydroxylase, were eluted from the column using a solution of 10%(w/v) α-methyl-D-mannoside. The eluate was used to immunize a New Zealand white rabbit. Alternatively, the eluate was run on a 10% SDS-polyacrylamide gel under non-reducing conditions and the 150,000 dalton dopamine β-hydroxylase Coomassie blue-stained band was excised and the protein electroeluted from the gel slice (Section 2.05.04.). This was also used for immunization.

As mentioned above, the resulting rabbit antisera to dopamine β-hydroxylase and chromogranin A were characterized by the immune-replica technique but their ability to immunoprecipitate the appropriate proteins remained to be tested. Chromaffin granule lysate proteins and the proteins from peak II comprising mainly dopamine β-hydroxylase were radio-iodinated by Dr. D.K. Apps using the chloramine T method. The antisera to chromogranin A and to dopamine β-hydroxylase were used to immunoprecipitate chromogranins from ^{125}I-labelled lysate proteins and dopamine β-hydroxylase from ^{125}I-labelled dopamine β-hydroxylase respectively as described in Section 2.03.12., and the immunoprecipitated proteins were examined by SDS-polyacrylamide gel electrophoresis (Section 2.04.01.) and autoradiography. The results, shown in Fig.3.03., indicated that the antiserum to chromogranin A was capable of immunoprecipitating

chromogranin A and several smaller chromogranins. No protein larger than chromogranin A, for example chromogranin B, was immunoprecipitated by this antiserum. Similarly, the antiserum to dopamine β-hydroxylase immunoprecipitated dopamine β-hydroxylase and several of the breakdown products derived from dopamine β-hydroxylase during the iodination procedure. In initial experiments, immunoprecipitations were performed in the absence of detergents according to Kessler (1981). Using this method about 98%+/-6% of the ^{125}I-labelled dopamine β-hydroxylase was immunoprecipitated but only 10%+/-4% of the ^{125}I-labelled chromogranins were immunoprecipitated. However, when immunoprecipitations were performed in the presence of 0.4%(w/v) SDS and 2%(w/v) Triton X-100 as recommended by Anderson and Blobel (1983) and described in Section 2.03.12., about 98%+/-4% of the ^{125}I-labelled chromogranins could be immunoprecipitated. Immunoprecipitations were routinely performed as described in Section 2.03.12..

The antisera were also examined for their cross-activity to ensure that the antiserum to chromogranin A did not contain antibodies to dopamine β-hydroxylase, and also that the antiserum to dopamine β-hydroxylase did not contain antibodies to chromogranin A. The results indicated that the antiserum to chromogranin A was specific for chromogranin A and several smaller chromogranins, and was not capable of immunoprecipitating dopamine β-hydroxylase (Fig. 3.04., lanes 2,7 and 9). Similarly, the antiserum produced against dopamine β-hydroxylase immunoprecipitated only dopamine β-hydroxylase and the smaller breakdown products, but it was incapable of immunoprecipitating chromogranin A or any of the lower molecular

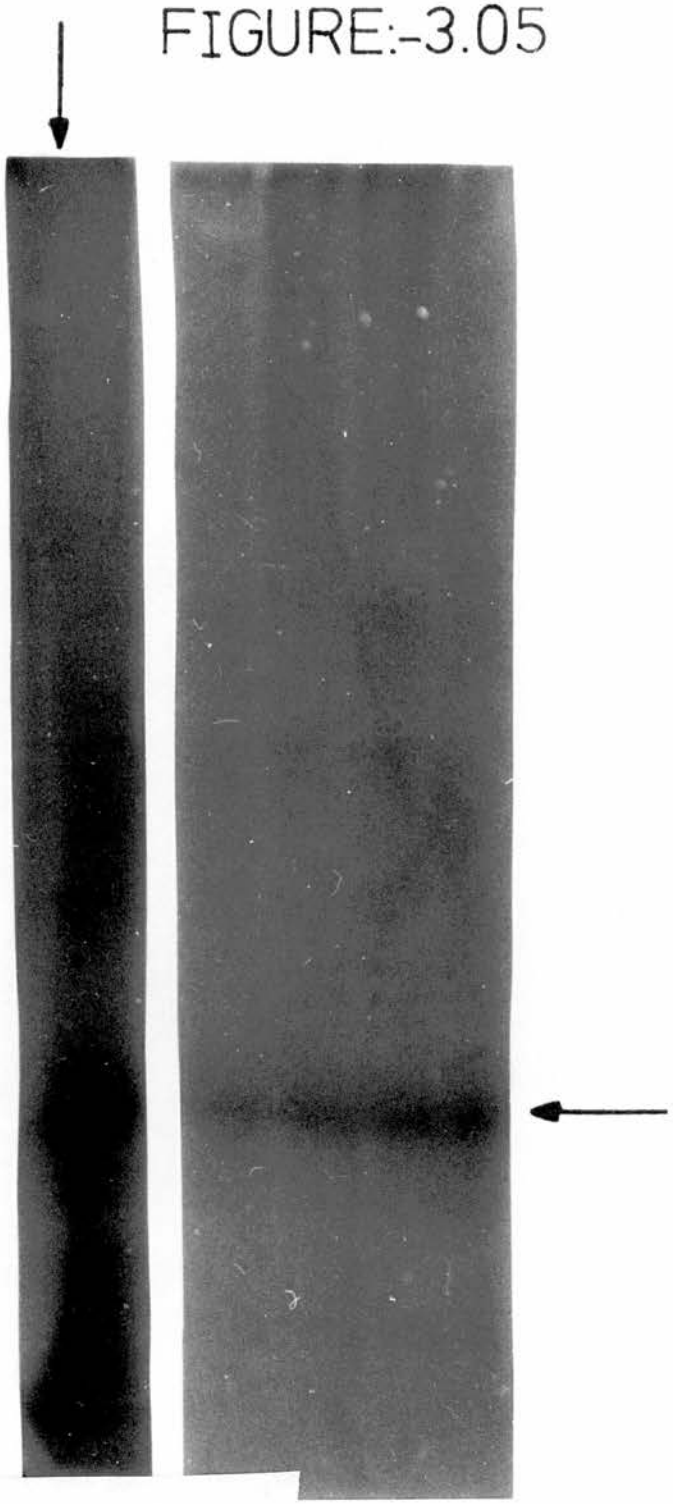
weight chromogranins (Fig. 3.04., lanes 1, 3 and 8).

Therefore, the antisera prepared against chromogranin A and dopamine β-hydroxylase specifically immunoprecipitated from a mixture of proteins, the proteins against which they had been prepared. It was fortuitous that the preparation of chromogranin A used to produce the antiserum had in fact been separated by DEAE-cellulose chromatography from the members of the chromogranin B family which at that time had still to be identified. This prevented confusion over the results obtained when the antiserum to chromogranin A was used to immunoprecipitate newly-synthesized polypeptides from *in vitro* translation reactions. Antiserum produced against a preparation of chromogranin A which has not been purified from the chromaffin granule matrix proteins by ion exchange chromatography will inevitably contain immunoglobulins against the chromogranin B family as explained by Falkensammer *et al.*, (1985a) and by Fischer-Colbrie and Frischenschlager (1985).

3.03.: The Antiserum to Cytochrome b_{561}

The method used to purify cytochrome b_{561} from chromaffin granule membranes is described by Apps *et al* (1980). Briefly, ammonium sulphate fractionation of detergent solubilized chromaffin granule membranes gave rise to a cytochrome enriched fraction (Section 2.02.05.) from which cytochrome was subsequently purified by chromatography on 6 amino-hexyl CL sepharose 4B (Section 2.02.05.). The fractions enriched with cytochrome b_{561}, as assessed by SDS-polyacrylamide gel electrophoresis, were pooled and lyophilized.

FIGURE:-3.05



Immunoprecipitation of

Cyt b_{561} from ^{125}I-Cyt b_{561}

This material was used to immunize a New Zealand white rabbit. Alternatively, this material was electrophoresed on a 12% SDS-polyacrylamide gel and the protein eluted from the excised Coomassie blue-stained cytochrome b_{561} band and used for immunization.

The resulting antiserum, when tested by the immune-replica technique, was specific for cytochrome and aggregates of cytochrome, even though antibodies were produced against only the cytoplasmically exposed domain of the protein (Abbs and Phillips, 1982; Hunter et al., 1982).

The ability of this antiserum to immunoprecipitate cytochrome b_{561} was tested using radio-iodinated cytochrome b_{561}, prepared by Dr. D.K. Apps, using the chloramine T method. Unfortunately, this protein does not iodinate well, possibly due to its hydrophobicity. However, enough ^{125}I-labelled cytochrome b_{561} was prepared to perform one set of immunoprecipitation reactions. As shown in Fig. 3.05., the antiserum produced against cytochrome b_{561} was capable of immunoprecipitating ^{125}I-labelled cytochrome b_{561}. When the immunoprecipitation was performed in the presence of 2%(w/v) Triton X-100 in the absence of SDS, approximately 15% of the total cytochrome was immunoprecipitated. Further attempts to ^{125}I-label cytochrome b_{561} were unsuccessful, and therefore no material was available to attempt the immunoprecipitation of ^{125}I-labelled cytochrome b_{561} using the method recommended by Anderson and Blobel (1983).

CHAPTER 4

OPTIMISATION OF THE TRANSLATION SYSTEMS

4.01.: TRANSLATION SYSTEMS

Several systems can be used to study the synthesis of proteins from isolated messenger RNA, each having its particular advantages and disadvantages. In this work, I have mainly used reticulocyte lysate (Section 4.01.01.), but some preliminary work using wheatgerm extract is also described (Section 4.05.)

4.01.01.: Reticulocyte Lysate

Rabbit reticulocyte lysate is one of the most commonly used cell-free systems for the translation of exogenous eukaryotic and viral messenger RNA (Hunt and Jackson, 1974). It is relatively easy to prepare and store (Section 2.03.01.) but the translation activity of individual reticulocyte lysate preparations varies considerably.

Rabbit reticulocyte lysate contains globin messenger RNA's and therefore has a high endogenous translation activity. This can be diminished by treatment with micrococcal nuclease in the presence of calcium ions (Pelham and Jackson, 1976) as described in Section 2.03.03.. An exogenously added messenger RNA molecule may be translated 40-70 times at a polypeptide elongation rate of almost one amino acid per second (Palmiter, 1973). Reticulocyte lysate produces very few incomplete polypeptides but it has been shown that significant amounts of endogenous messenger RNA fragments remain after the micrococcal nuclease digestion, including sequences which contain initiation sites (Kay and Benzie, 1982). Added messenger RNA's must therefore compete with these fragments for ribosome

binding.

Reticulocyte lysate has negligible ribonuclease activity due to the presence of endogenous ribonuclease inhibitor, but it is uniquely sensitive to certain inhibitors such as double-stranded messenger RNA and oxidised thiol compounds (Pelham and Jackson, 1976; reviewed in Maitra et al., 1982). Furthermore presence of an endogenous pool of amino acids may result in decreased incorporation of added labelled amino acid into protein, and the reticulocyte lysate's stringent requirement for haemin makes it atypical for use in studies of translational control mechanisms. Haemin is a suppressor of an inhibitor of the initiation factor eIF2. This inhibitor acts by phosphorylating the 38K subunit of eIF2, rendering it incapable of interaction with protein factors which enhance or modulate the functioning of eIF2 (Farrell et al., 1977). Consequently the addition of haemin prolongs linear protein synthesis.

Reticulocyte lysate contains a soluble protein, functionally equivalent to the signal recognition particle (SRP), since translocation of a secretory protein across salt-washed rough microsomes, which therefore lack SRP, can occur when used in conjunction with reticulocyte lysate (Meyer et al., 1982). This suggests that reticulocyte lysate may be an unsuitable translation system for the study of proteins synthesized as precursors, since the SRP functions in vitro to selectively halt the translation of those nascent proteins (Walter and Blobel, 1981). However, there have been no further reports of disadvantages of reticulocyte lysate for such studies. In fact, recently it has been suggested that the

elongation arrest attributed to the SRP is an artefact of wheatgerm extract, and that in mammalian cell-free translation systems, the SRP does not halt translation (Walter, 1985).

4.02.01.: The Translation Reaction and its Optimisation

The reticulocyte lysate must be supplied with an energy-generating system such as creatine phosphate/creatine kinase. In addition, 19 unlabelled amino acids, dithiothreitol (which preserves cysteine and methionine in their reduced forms), spermidine (a polyamine which may enhance protein synthesis) and HEPES buffer (pH7) are added as a translation "cocktail" (Section 2.03.02.).

Reticulocyte lysates usually require optimal magnesium and potassium ion concentrations of around 0.6mM and 80mM respectively (Hunt and Jackson, 1974). However, these optima vary from one preparation to another and should be determined for each lysate preparation. Magnesium and potassium ion concentration optima were determined for the translation of reticulocyte endogenous messenger RNA and also for exogenously added adrenal medullary poly-A^+ RNA.

4.02.02.: Optimisation of $[Mg^{2+}]$ for the Translation of Endogenous Reticulocyte messenger RNA

Translations of endogenous reticulocyte messenger RNA were performed in the presence of 77mM K^+ and the concentrations of Mg^{2+} were varied by adding an equal volume of various stock solutions of magnesium acetate to the translation reaction (Section 2.03.02.).

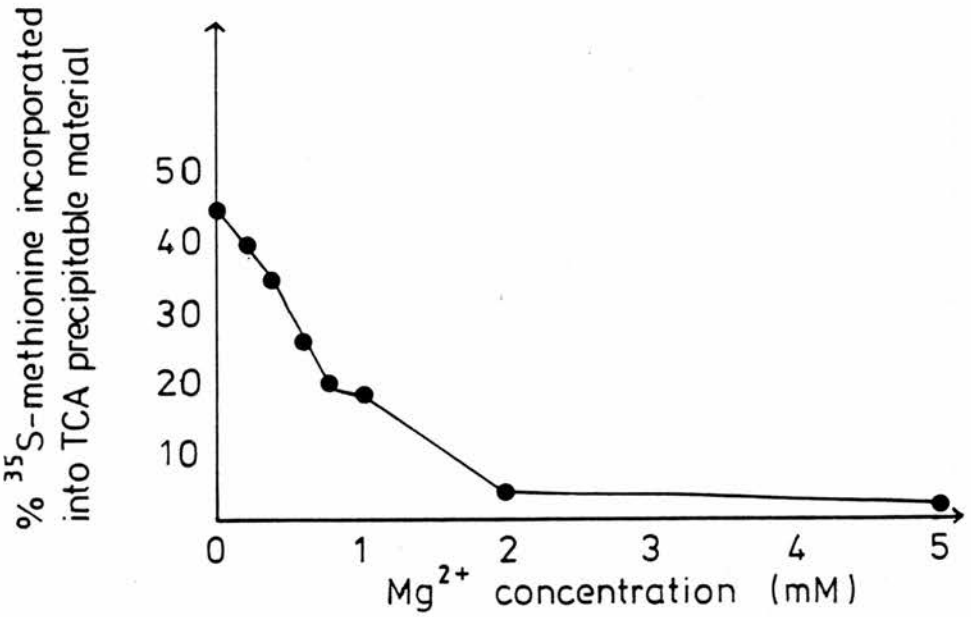


Fig:- 4.02.01.:% ^{35}S-methionine incorporated into TCA - precipitable material versus Mg^{2+} concentration in reticulocyte lysate translations using endogenous mRNA (incubations for 60 minutes at 30°C)

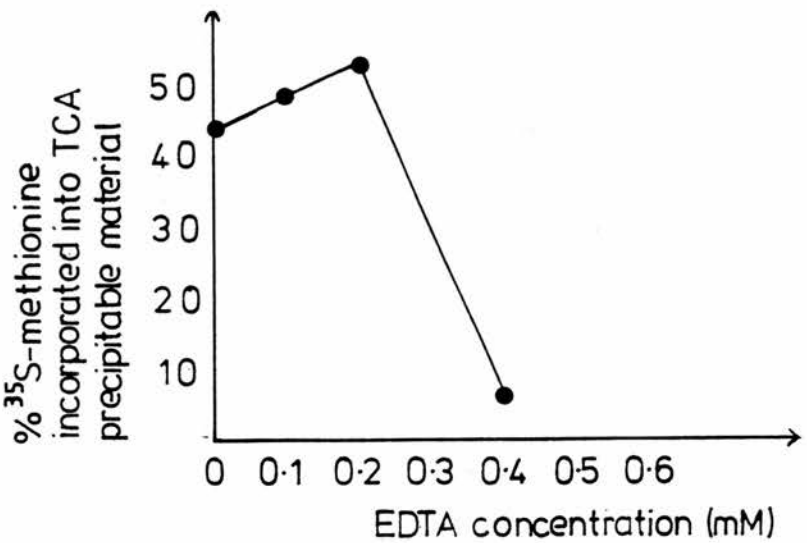


Fig:- 4. 02.02.% ^{35}S-methionine incorporated into TCA - precipitable material versus EDTA concentration in reticulocyte lysate translations using endogenous mRNA (incubations for 60 minutes at 30°C)

The percentage of ^{35}S-methionine incorporated into TCA-precipitable material after incubating the translation mixes at 30°C for 60 minutes was determined by treating 2x1µl samples as described in Section 2.03.05.. The results obtained are shown in Fig. 4.02.01.. Surprisingly, maximum incorporation of radiolabel occurred when no Mg^{2+} was added. Consequently the effect of varying the concentration of EDTA (pH7.4) without added Mg^{2+} was investigated. Translations were performed in the presence of various concentrations of EDTA. The results, Fig. 4.02.02., show maximum incorporation of ^{35}S-methionine into TCA-precipitable counts when 0.2mM EDTA was present in the translation reaction. At this concentration of EDTA, over 50% of ^{35}S-methionine available was incorporated into TCA-precipitable material. The efficiency of ^{35}S-methionine incorporation into TCA-precipitable material using the reticulocyte endogenous messenger RNA can be used as an indicator of how actively the message-dependent reticulocyte lysate will translate exogenously-added messenger RNA.

4.02.03.: Optimisation of $[K^+]$ for the Translation of Endogenous Reticulocyte messenger RNA

Translations of endogenous reticulocyte messenger RNA were performed in the presence of 0.2mM EDTA and the K^+ concentration was varied by adding an equal volume of various stock solutions of potassium chloride. After incubation at 30°C for 60minutes, the percentage of ^{35}S-methionine incorporated into TCA-precipitable material was calculated as described in Section 2.03.05.. The incorporation of radiolabel was maximal when the translations were

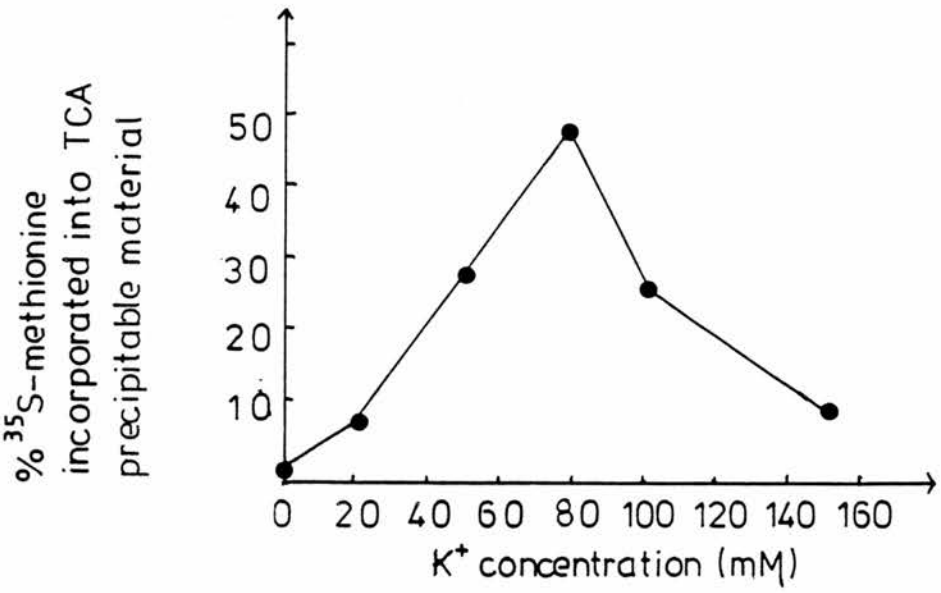


Fig. : - 4.02.03. : % ^{35}S-methionine incorporated into TCA-precipitable material versus K^+ concentration in reticulocyte lysate translations using endogenous mRNA (incubations for 60minutes at 30°C)

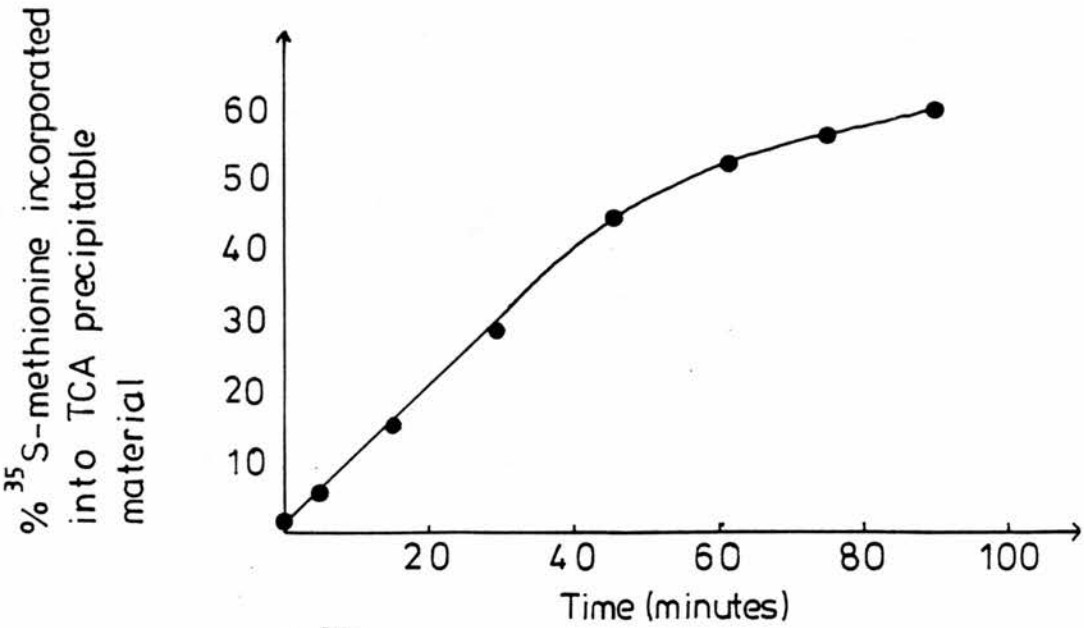


Fig. : - 4.02.04. : % ^{35}S-methionine incorporated into TCA-precipitable material versus length of incubation (minutes) in reticulocyte lysate translations using endogenous mRNA

performed in the presence of 77mM K^+ as shown in Fig.4.02.03.

4.02.04.: Protein Synthesis as a Function of Time using Endogenous Reticulocyte messenger RNA

Using the optimal concentrations of Mg^{2+} and K^+, translation of endogenous reticulocyte messenger RNA was performed and samples (2x1µl) were taken and processed to determine the extent of protein synthesis at different time intervals after initiating the reaction by starting incubation at 30°C. A plot of the percentage of ^{35}S-methionine incorporated into TCA-precipitable material (Fig.4.02.04.) showed that incorporation of radioactive methionine occurred at a constant rate for about 45 minutes. Translations were routinely incubated at 30°C for 60 minutes.

Once conditions had been optimised for the translation of endogenous reticulocyte lysate messenger RNA, conditions had to be optimised for the translation of exogenously added adrenal medullary messenger RNA using micrococcal nuclease-treated, message-dependent reticulocyte lysate.

4.03.01.: Optimisation of $[Mg^{2+}]$ and $[K^+]$ for the translation of adrenal medullary messenger RNA

Experiments, similar to those described in Sections 4.02.02.-4.02.04. were performed using message-dependent reticulocyte lysate (Section 2.03.03.) and adrenal medullary poly A^+ RNA (Section

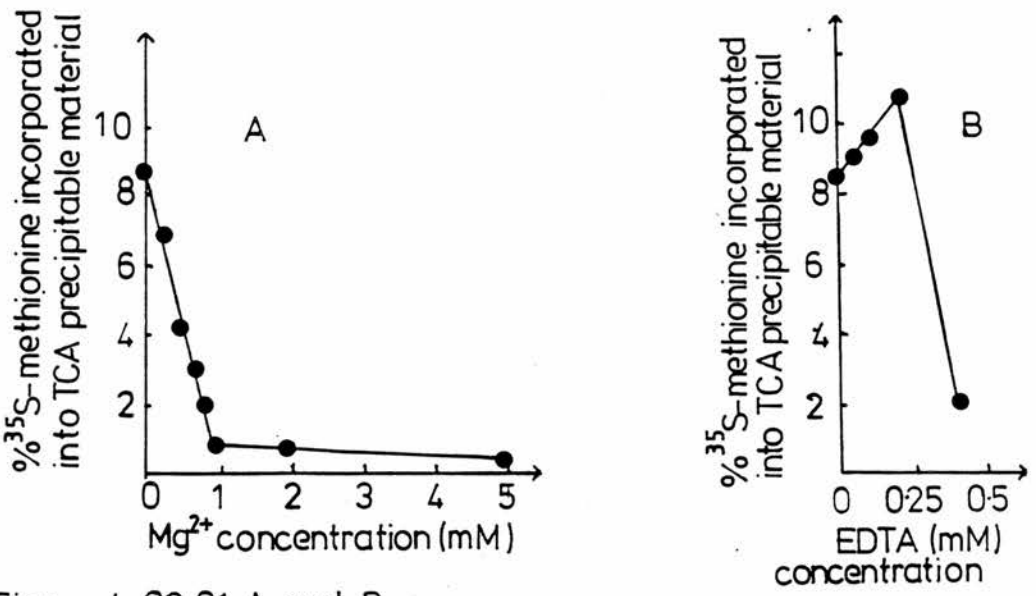


Fig.: - 4.03.01. A and B
%^{35}S-methionine incorporated into TCA-precipitable material versus A) Magnesium^{2+} and B) EDTA concentration in message-dependent reticulocyte lysate translations of adrenal medullary mRNA (incubations for 60 minutes at 30°C)

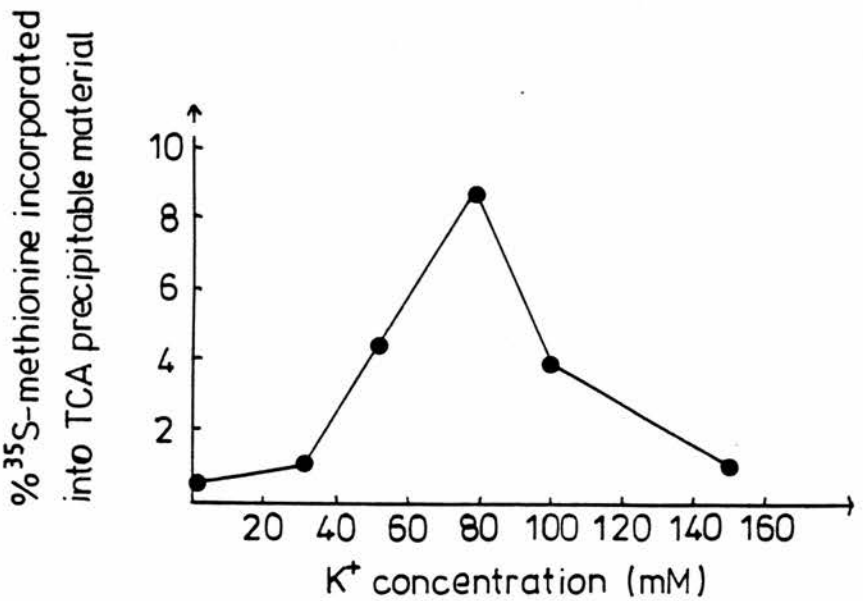


Fig.: - 4.03.02. : %^{35}S-methionine incorporated into TCA-precipitable material versus K^+ concentration in message-dependent reticulocyte lysate translations of adrenal medullary mRNA (incubations for 60 minutes at 30°C)

2.02.10.). The effects of different Mg^{2+} and K^{+} concentrations on the incorporation of radiolabel into TCA-precipitable counts are shown in Figs.4.03.01A and 4.03.02. respectively. The optimal [K^{+}] for the translation of adrenal medullary messenger RNA is 77mM, identical to that for the translation of endogenous reticulocyte messenger RNA. Similarly, maximal incorporation of radiolabel was obtained at 0mM Mg^{2+} when translating adrenal medullary messenger RNA in message-dependent reticulocyte lysate and therefore the effect of EDTA on the incorporation of radiolabel was investigated. The result, shown in Fig.4.03.01B., indicated that maximal protein synthesis occurred at 0.2mM EDTA, identical to the result obtained for the translation of endogenous reticulocyte messenger RNA.

The requirement for EDTA is an unexpected result and is not a peculiarity of this one preparation of reticulocyte lysate; four preparations of reticulocyte lysate have had identical optimal EDTA and K^{+} requirements for the translation of endogenous and exogenous messenger RNA. The EDTA is replacable by EGTA. It is possible that the EDTA and EGTA chelate contaminating heavy metal ions, from an unknown source, which severely impair the activity of the reticulocyte lysate.

4.03.02.: Determination of the Optimal poly A^{+} RNA concentration in a translation reaction

Translation reaction mixtures were prepared as described in Section 2.03.04.. Identical volumes of various concentrations of poly A^{+} RNA were added to each translation immediately before

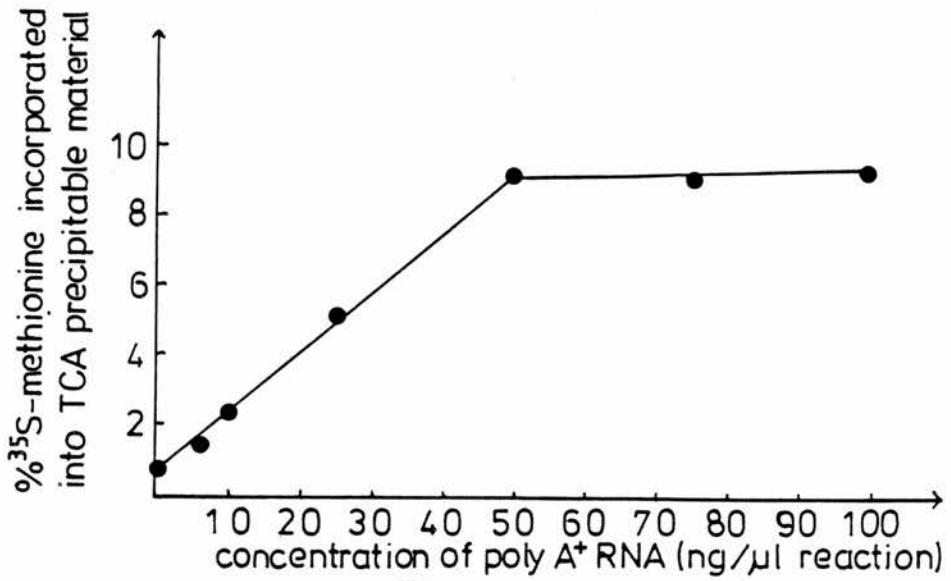


Fig.: -4.03.03.: % ^{35}S-methionine incorporated into TCA-precipitable material versus concentration of adrenal medullary poly A$^+$ RNA in message-dependent reticulocyte lysate translations (incubations for 60minutes at 30°C)

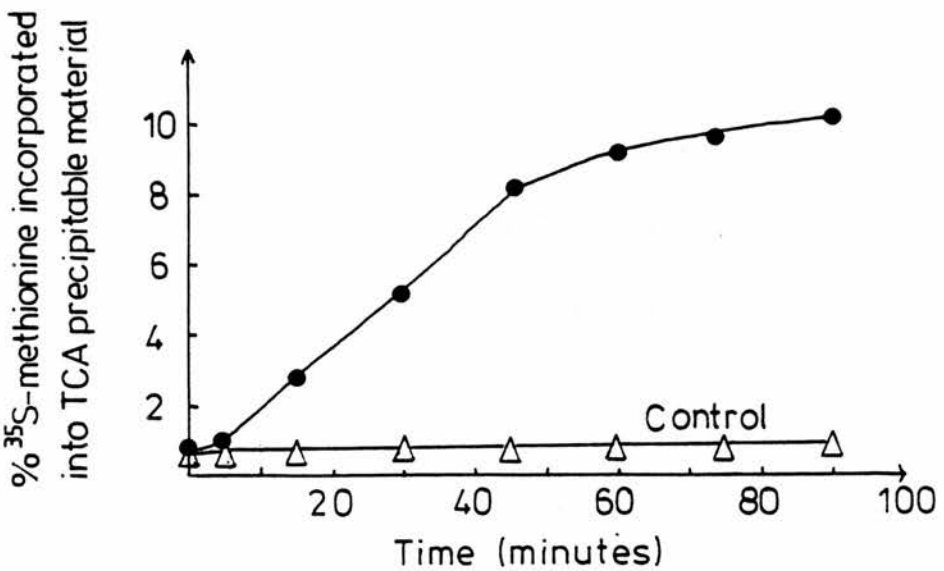


Fig.: - 4.03.04 : % ^{35}S-methionine incorporated into TCA-precipitable material versus length of incubation (minutes) using message-dependent reticulocyte lysate to translate adrenal medullary mRNA (Control without added mRNA)

initiating the reaction by transferring them from ice to 30°C. The final concentration of poly A^+ RNA in the translation reactions varied between 0ng/µl and 100ng/µl. After incubation for 60 minutes at 30°C, samples were taken and processed (Section 2.03.05.) to determine the amount of radiolabel incorporated. As shown in Fig. 4.03.03., the optimal concentration appears to be 50ng of adrenal medullary poly A^+ RNA per µl of translation reaction.

4.03.03.: Protein Synthesis as a Function of Time using Message-Dependent Reticulocyte Lysate to Translate Adrenal Medullary messenger RNA

Translation of adrenal medullary messenger RNA by message-dependent reticulocyte lysate was performed as described in 2.03.04.. Samples (2x1µl) were removed at intervals after the start of the reaction and the extent of protein synthesis was determined as described in Section 2.03.05.. A control experiment was run in the absence of added poly A^+ RNA to determine the extent of ^{35}S-methionine incorporation into TCA-precipitable material by micrococcal nuclease treated reticulocyte lysate. The results are shown in Fig.4.03.04.. In this system, protein synthesis is linear for a period of 45 minutes. There is no significant protein synthesis in the control, and therefore micrococcal nuclease-treated reticulocyte lysate is completely dependent on exogenously added messenger RNA. The optimal conditions for the translation of adrenal medullary messenger RNA in reticulocyte lysate are summarized in Fig.4.03.05..

Fig.:- 4.03.05.

Final concentration of components in a translation reaction using reticulocyte lysate.

25 µM of 19 amino acids

385-580µCi ^{35}S-methionine/ml

77mM KCl

0·2mM EDTA

0·5mM Spermidine

7·7mM Creatine Phosphate

30µg/ml Creatine Kinase

1·9mM Dithiothreitol

19·2mM HEPES

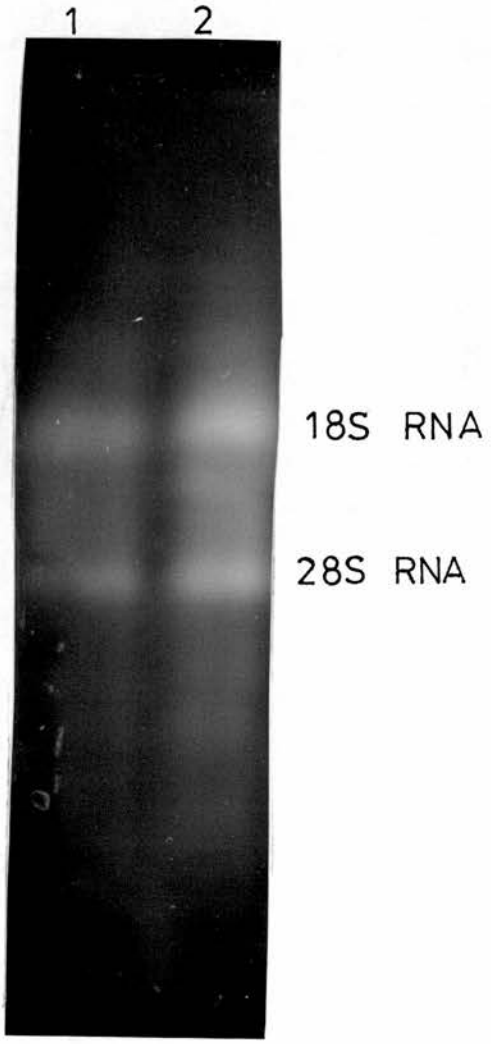
50 ng poly A^+ RNA / µl translation reaction

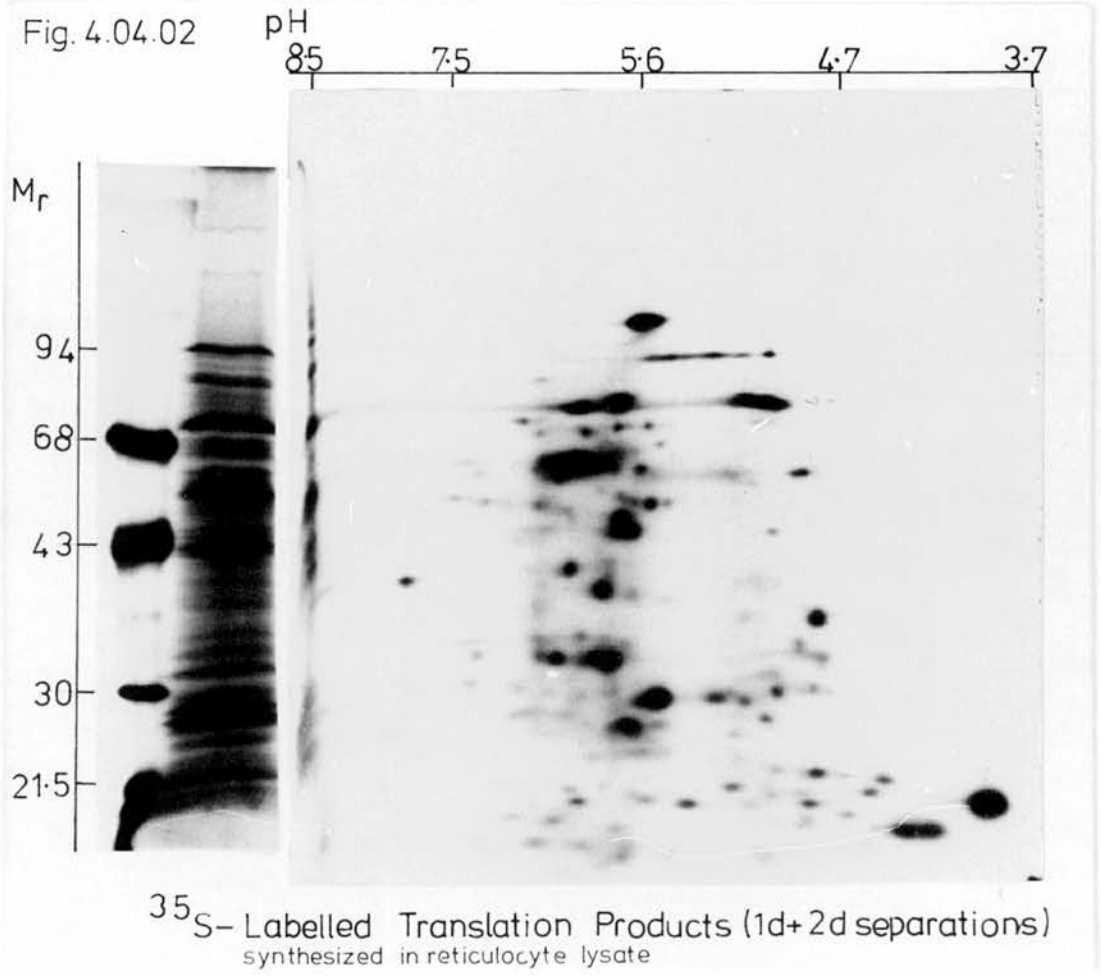
60% v/v nuclease treated reticulocyte lysate

FIGURE 4.04.01.: Ethidium bromide-stained agarose gel (1% w/v) of adrenal medullary messenger RNA.

Lane 1 : 10µg of poly A^+ RNA

Lane 2 : 20µg of poly A^+ RNA





Fluorograph of the radioactively-labelled polypeptides synthesized when reticulocyte lysate was supplemented with adrenal medullary messenger RNA. Approximately 10^6 **dpm of TCA-precipitable material was loaded per gel and fluorographs were exposed for 4 days at -70°C.**

Translation of adrenal medullary messenger RNA for a period of 60 minutes results in the incorporation of about 10% of the available ^{35}S-methionine into TCA-precipitable material. This is about 20% of the efficiency of the incorporation during translation of endogenous reticulocyte messenger RNA. This lower efficiency is typical (Kay and Benzie, 1982). Initiation is the rate-limiting step of translation (Bergman and Lodish, 1979). If adrenal medullary messenger RNA molecules are weak initiators, either naturally or due to damage of initiation sequences during preparation, they will be translated less efficiently than strongly initiating messenger RNA species such as β-globin messenger RNA (Kabat and Chappell, 1977). Also any residual β-globin messenger RNA initiation sequences remaining after nuclease treatment of the reticulocyte lysate will compete against exogenous messenger RNA for initiation factors and ribosomal subunits (Kay and Benzie, 1982). Micrococcal nuclease treatment of reticulocyte lysate causes some fragmentation of ribosomal RNA and this also decreases the translation activity (Kennedy et al., 1981).

4.04.01.: Agarose gel analysis of isolated poly A^+ RNA

Poly A^+ RNA was analysed on a 1%(w/v) agarose gel containing formaldehyde (Section 2.04.07.). The gel was stained with ethidium bromide and visualized under ultraviolet light (Fig.4.04.01.). The poly A^+ RNA appeared as a smear, suggesting a range of different sizes of RNA molecules. There was also some contaminating ribosomal RNA despite purification of the poly A^+ RNA by affinity chromatography (Section 2.02.10.). However, the intactness of the

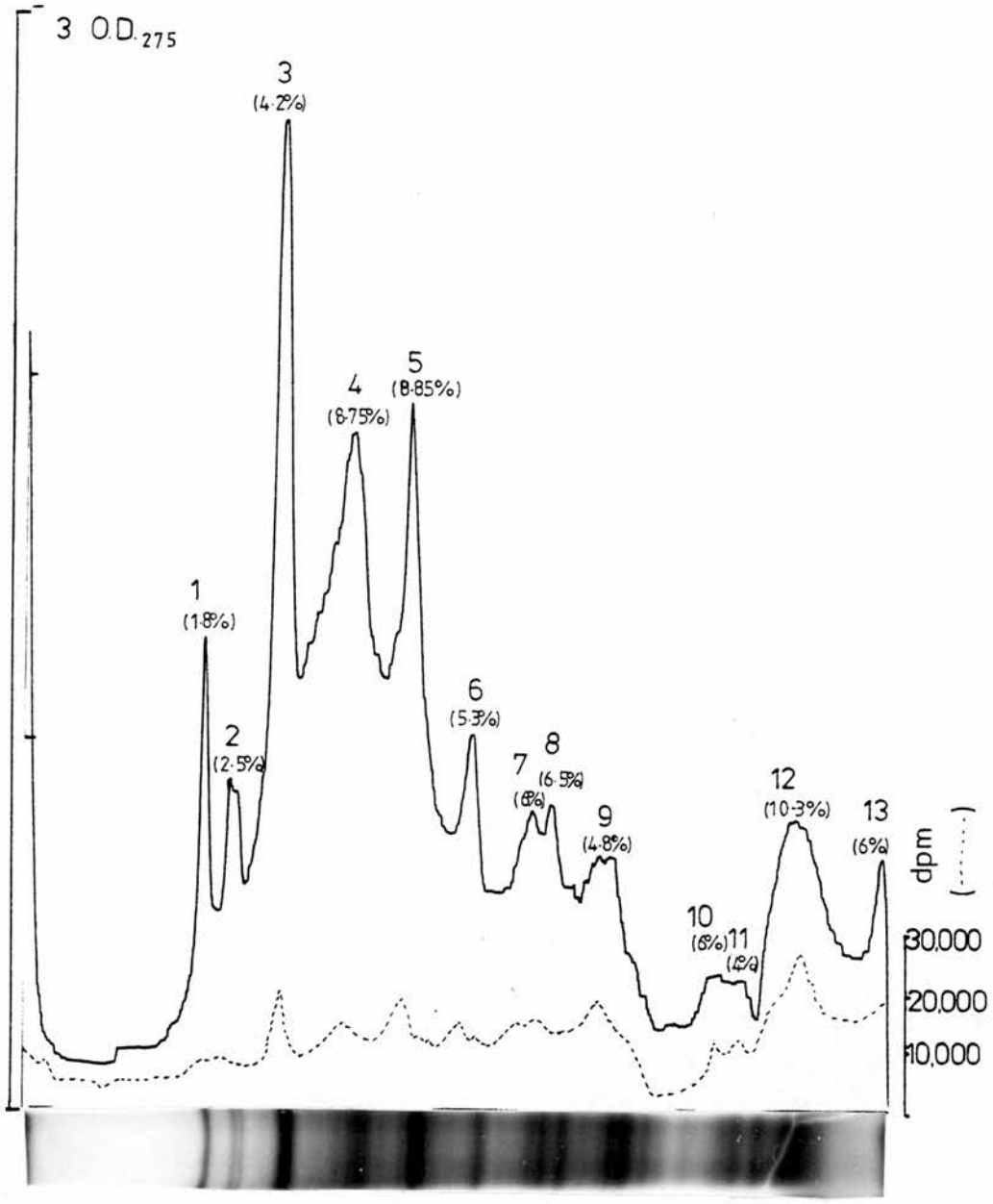


FIGURE 4.04.03.: DENSITOMETER SCAN OF THE FLUOROGRAPH OF A GEL TRACK AND PROFILE OF THE RADIOACTIVITY ELUTED FROM A SIMILAR GEL TRACK. ADRENAL MEDULLARY MESSENGER RNA WAS TRANSLATED IN RETICULOCYTE LYSATE. ABOUT 10^6 DPM OF TCA-PRECIPITABLE MATERIAL WAS LOADED PER WELL ON AN 8-15% SDS-POLYACRYLAMIDE GEL. THIRTEEN PEAKS ARE NUMBERED AND THE PERCENTAGE OF THE TOTAL RADIOACTIVITY UNDER EACH PEAK IS INDICATED IN BRACKETS.

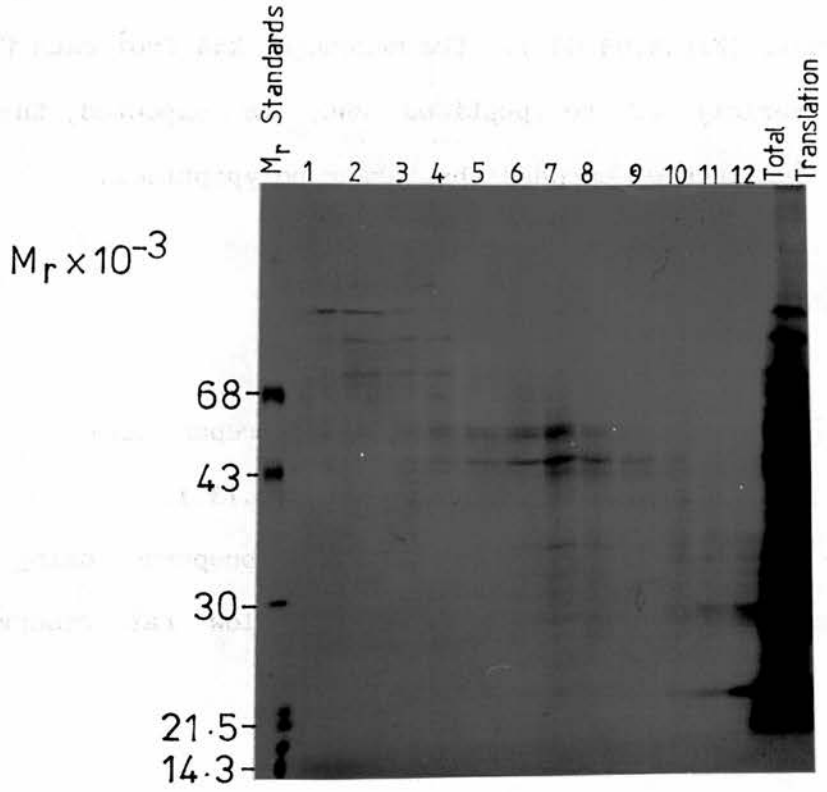
ribosomal RNA indicated that there was little ribonuclease contamination of the final RNA preparation.

4.04.02.: Examination of Translation Products

The polypeptides synthesized from a translation of adrenal medullary messenger RNA were examined by one- and two-dimensional SDS-polyacrylamide gel electrophoresis (Section 2.04.04.) followed by fluorography (Section 2.04.02.). As shown in Fig 4.04.02., the translation system is capable of synthesizing a large range of different polypeptides with molecular weights up to around 150,000 daltons. Additional quantitative information about the translation products was obtained from a densitometer scan of a fluorographed one-dimensional gel lane, and from counting the radioactivity eluted from 2mm gel slices by liquid scintillation counting (Section 2.05.04.). As shown in Fig.4.04.03., there are 13 major peaks of radioactivity, however most peaks contain several distinct radioactively labelled polypeptides as seen by comparison with the fluorograph.

Two-dimensional SDS-polyacrylamide gel analysis of the translation products reveals more information about the variety of polypeptides synthesized. As shown in Fig.4.04.02., between 40 and 50 polypeptides, of which 12 are major products, are easily visualized by fluorography after a two-dimensional separation of the radioactively labelled translation products from a 15µl translation reaction.

FIGURE 4.04.04.: Translation of the various fractions of adrenal medullary poly A^+ RNA obtained by centrifugation of the RNA through 5-30% (w/v) sucrose gradients.



4.04.03.: SDS-polyacrylamide Gel Analysis of the Translation Products from poly A^+ Fractions Obtained from Density Gradient Centrifugation

Poly A^+ RNA (300μg) was separated on 5-30%(w/v) sucrose gradients (Section 2.05.03.). Fractions of 0.3ml were collected, made 300mM in NaCl, and RNA was precipitated (Section 2.02.11.) three times in order to fully remove residual SDS. The messenger RNA from each fraction was translated in message-dependent reticulocyte lysate and the translation products separated by SDS-polyacrylamide gel electrophoresis (Fig.4.04.04.). The messenger RNA from each fraction encoded a variety of polypeptides and, as expected, the larger messenger RNA molecules encoded the larger polypeptides.

4.05.01.: Wheatgerm Extract

Wheatgerm extract is cheap and easy to prepare as described by Marcus et al. (1974) (Section 2.03.13.). The most translation-efficient wheatgerm extracts are prepared using young, fresh wheatgerm, possibly due to its low fat reserves (G. Falkensammer, personal communication).

Wheatgerm extract has low endogenous translation activity (Roberts and Paterson, 1973) and there is no need therefore, for micrococcal nuclease treatment. The endogenous pool of amino acids is also low, thus ensuring high incorporation of added labelled amino acid. Wheatgerm extract has some protease and ribonuclease activities which must be inhibited, but the main problem with this translation system is its tendency to produce incomplete polypeptides

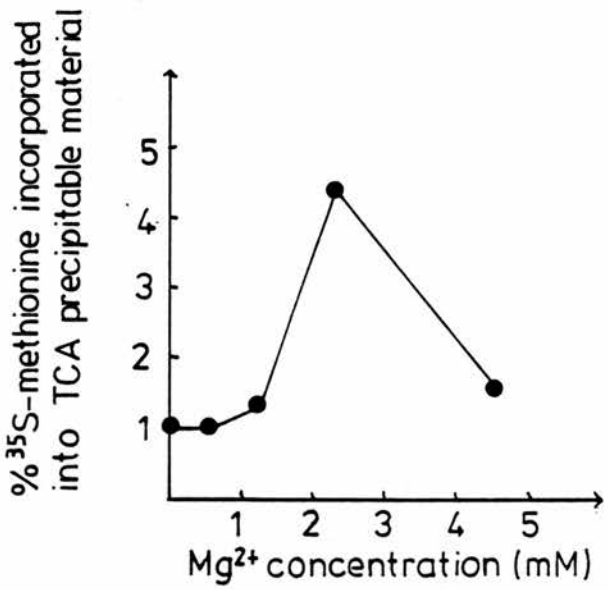


Fig.:- 4.05.01.: % ^{35}S- methionine incorporated into TCA-precipitable material versus Mg^{2+} concentration in Wheatgerm extract translations of adrenal medullary mRNA (incubations for 60 minutes at 25°C)

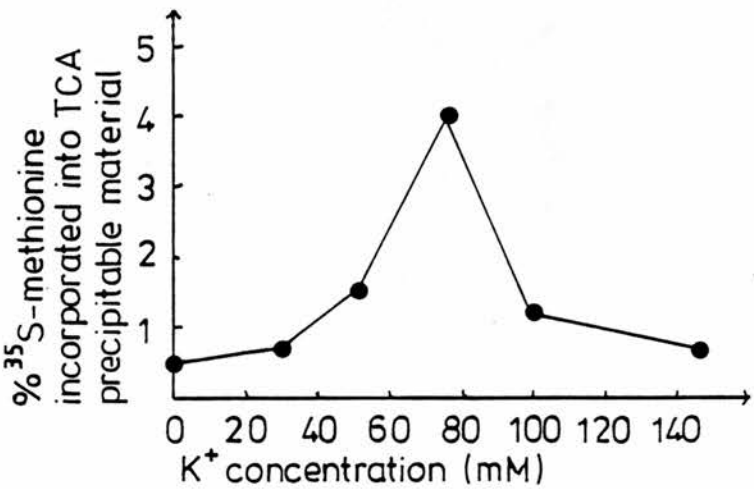


Fig.:- 4.05.02.: % ^{35}S- methionine incorporated into TCA-precipitable material versus K^{+} concentration in Wheatgerm extract translations of adrenal medullary mRNA (incubations for 60 minutes at 25°C)

due to premature termination. Polyamines help overcome this problem by stimulating the rate of polypeptide chain elongation. Approximately 5 rounds of translation are completed per messenger RNA molecule.

4.05.02.: The Translation of Adrenal Medullary messenger RNA in the S-30 Wheatgerm Translation System

Translations performed in wheatgerm extract have similar requirements to those performed in reticulocyte lysate. The translation reaction must be supplied with an energy-generating system, one radioactively labelled amino acid (^{35}S-methionine) and 19 unlabelled amino acids, dithiothreitol, and poly A^+ RNA, Mg^{2+} and K^+, the optimal concentrations of which must be determined in a similar manner to that used for translations with reticulocyte lysate. The wheatgerm translation reaction mixture was set up as described in Section 2.03.14.. Mg^{2+} and K^+ optima were determined to be 2.25mM and 77mM respectively as shown in Figs.4.05.01 and 4.05.02. respectively. The wheatgerm extract was prepared in a different laboratory, in the Edinburgh University Botany Department, using their water and chemicals. This may account for the wheatgerm extract having a Mg^{2+} optimum instead of a requirement for EDTA. The optimal concentration of adrenal medullary poly A^+ RNA was 50ng/µl of translation reaction. Protein synthesis was linear for 45 minutes at 25°C, but translations were routinely performed for 60 minutes at 25°C, and about 5% of the available ^{35}S-methionine was incorporated into TCA-precipitable material. Protein synthesis in the absence of exogenously added poly A^+ RNA was negligible (<1%).

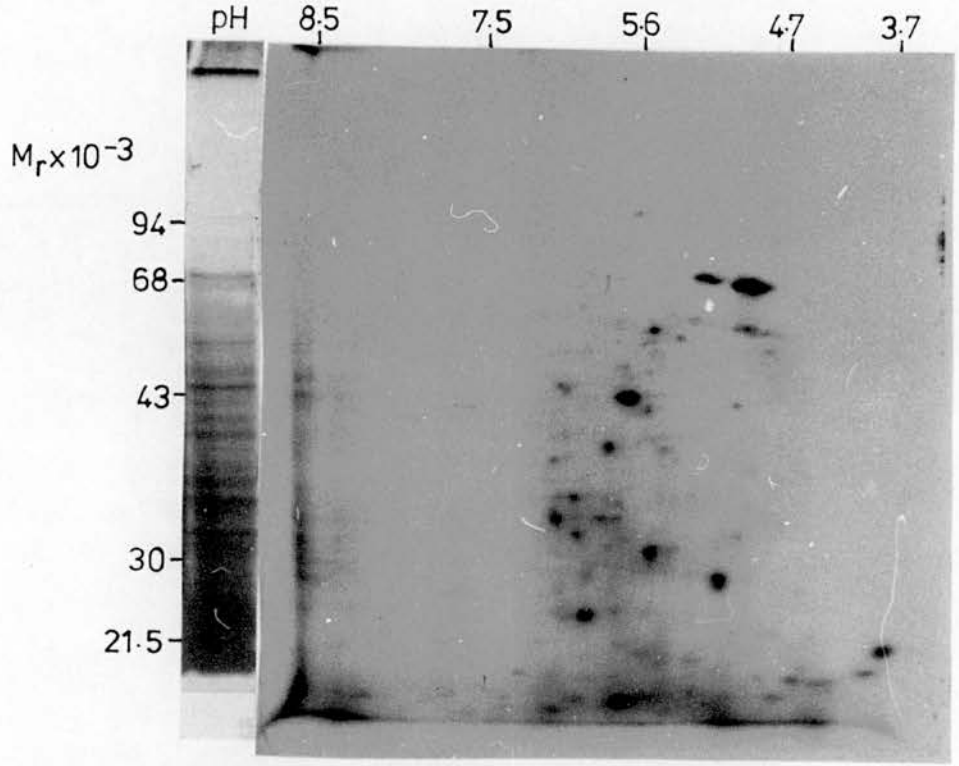


Fig. 4.05.03. : ^{35}S-labelled translation products synthesized in wheatgerm extract

Fluorograph of the radioactively-labelled polypeptides synthesized when wheatgerm extract was supplemented with adrenal medullary messenger RNA. Fluorograph was exposed for 4 days at -70°C.

Examination of the translation products by one- and two-dimensional SDS-polyacrylamide gel electrophoresis followed by fluorography (Fig. 4.05.03.), revealed that this wheatgerm translation system did not synthesize high molecular weight polypeptides with the same efficiency as reticulocyte lysate. Human placental ribonuclease inhibitor (Scheele and Blackburn, 1979) was added to wheatgerm translations of adrenal medullary messenger RNA, in an attempt to inhibit any endogenous ribonuclease in the wheatgerm extract but neither the percentage of ^{35}S-methionine incorporated into TCA-precipitable material, nor the molecular weight range of the translation products increased. The problem was probably due to endogenous wheatgerm extract proteases. Reticulocyte lysate was, in this instance, a better translation system than the wheatgerm S-30 system. Also, in practice, the reticulocyte lysate is more suitable for studies of cotranslational processing using canine pancreas microsomes (Scheele *et al.*, 1980).

4.06.01.:Cotranslational Processing *in vitro*

Cotranslational processing events can be reproduced *in vitro* by supplementing the cell free translation system with rough microsomal membranes (Blobel and Dobberstein, 1975a; Katz *et al.*, 1977; Shields and Blobel, 1978). Canine pancreas rough microsomes are often employed in such studies owing to their low levels of ribonuclease activity (Scheele *et al.*, 1980). Since processing events are cotranslational, the microsomal membranes must be present during protein synthesis (Blobel and Dobberstein, 1975a).

FIGURE 4.06.01.: Dog pancreas microsomal proteins, separated by SDS-polyacrylamide gel electrophoresis, were transferred to nitrocellulose paper and probed with antiserum to dog pancreas docking protein.

Lane 1 : Molecular weight standards

Lane 2 : Immune replica as described above

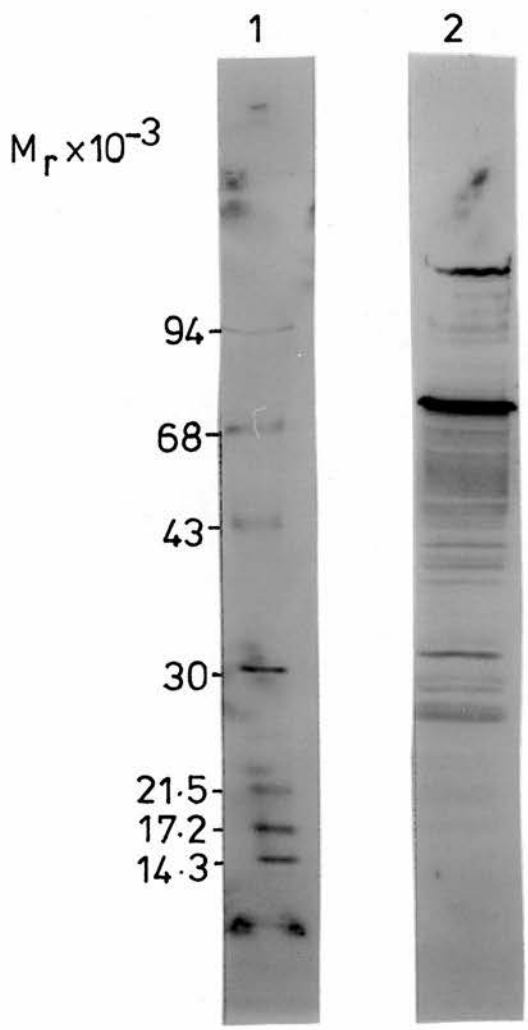
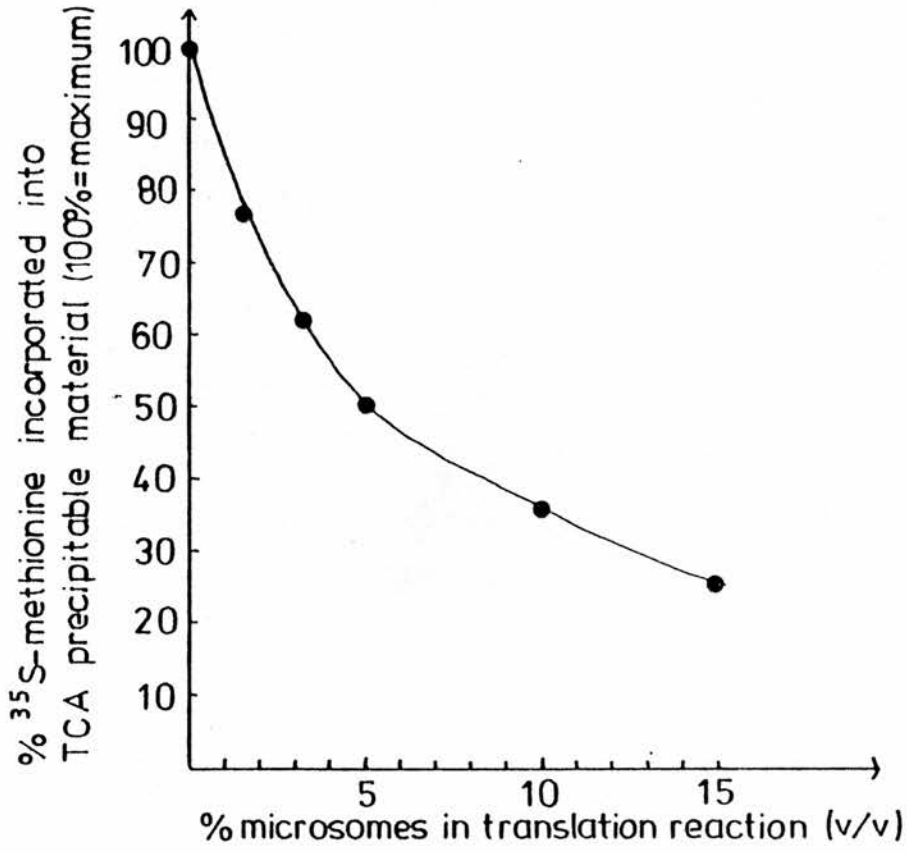


Fig.:- 4.06.02. % of maximum ^{35}S-methionine incorporated into TCA-precipitable material versus % dog pancreas microsomes in the translation reaction (v/v)



Dog pancreas rough microsomes were prepared according to Walter and Blobel, (1983b), as described in Section 2.03.06., and their endogenous messenger RNA was destroyed immediately before use by micrococcal nuclease treatment as described in Section 2.03.07.. No endogenous messenger RNA was detected when the nuclease-treated dog pancreas rough microsomes were assayed in the reticulocyte lysate translation system. Translocation across the endoplasmic reticulum occurs following attachment of ribosomes, messenger RNA and signal recognition particle to the docking protein on the cytosolic surface of the membrane (Section 1.02.01.). The presence of this protein in the canine pancreas rough microsome preparation was tested by an immune replica of a one-dimensional SDS-polyacrylamide gel separation of the microsomal proteins using an antiserum directed against dog pancreas docking protein (gift from D. Meyer). The resulting autoradiograph (Fig.4.06.01.) revealed the presence of the 70,000 dalton polypeptide.

Translations of messenger RNA in the presence of dog pancreas rough microsomes were performed by adding nuclease-treated microsomes to a final concentration of $5A_{260}$ units/ml of translation reaction, immediately before the addition of the poly A^+ RNA. The processing of polypeptides is optimal at this concentration of rough microsomes (Rothman and Lodish, 1977). However, the high concentrations of microsomal membranes, which are necessary to drive processing reactions to completion, inhibit overall protein synthesis (Fig. 4.06.02.). The reason for this inhibition, which is more severe in wheatgerm extract (Dobberstein and Blobel, 1977) is unknown (Shields

^{35}S-labelled Proteins in the microsomal pellet from a translation in the presence of dog pancreas microsomes.

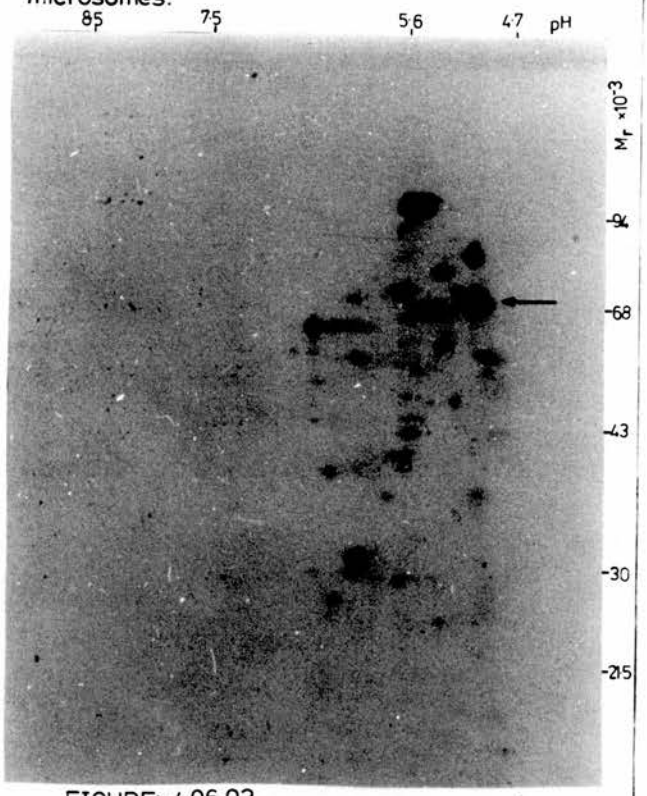


FIGURE:-4.06.03.

^{35}S-labelled Proteins remaining in the supernatant from a translation in the presence of dog pancreas microsomes

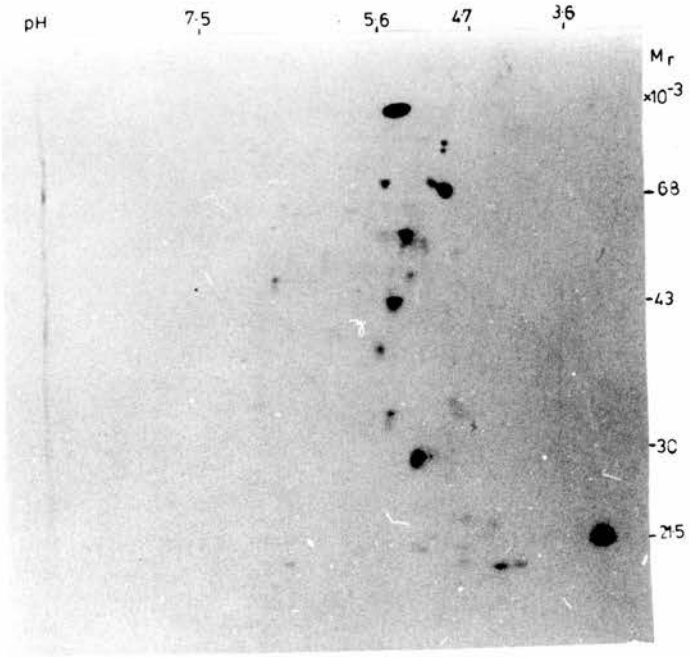


FIGURE:- 4.06.04.

and Blobel, 1978; Scheele et al., 1980). After incubation for 60 minutes at 30°C, the microsomes were pelleted by centrifugation. The supernatant, containing unprocessed, newly-synthesized polypeptides was usually discarded. The microsomal pellet, containing newly synthesized polypeptides which had been processed, was resuspended and washed in 0.15M sucrose, 10mM HEPES pH7, 1mM PMSF. The washed microsomal pellet was resuspended in the appropriate gel sample buffer for gel analysis. Two-dimensional gel analysis of the processed polypeptides associated with the microsomal pellet (Fig. 4.06.03.) and of the unprocessed polypeptides which remain in the supernatant (Fig. 4.06.04.), suggest that adrenal medullary messenger RNA encodes several polypeptides which are translocated across microsomal membranes. Confirmation of this was provided by the experiments described in Sections 4.06.02 and 4.06.03..

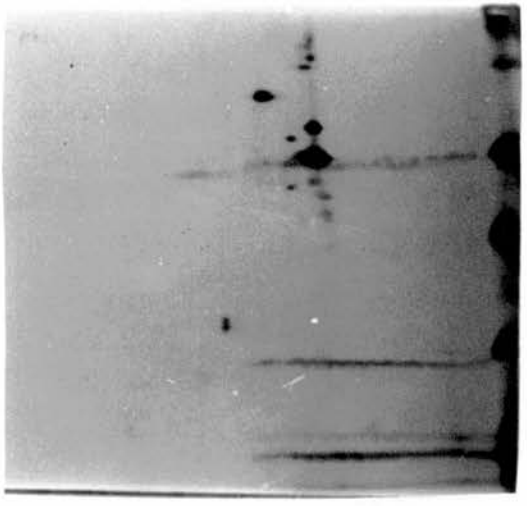
4.06.02.: Post-translational Proteolysis Assay

Functional dog pancreas microsomes offer protection to translocated polypeptides from exogenously added proteases and this property is used as the assay for a microsome preparation that is functional in translocation (Scheele et al., 1980; Morimoto et al., 1983). The method (Section 2.03.09.) involves the addition of exogenous proteases, to a translation reaction performed in the presence of nuclease-treated dog pancreas microsomes, and analysis of the products by gel electrophoresis. Any newly-synthesized polypeptides which have been translocated across the membranes will be protected from the proteases and remain undegraded.

FIGURES 4.06.05. and 4.06.06.

The post-translational proteolysis assay was performed exactly as described in Section 2.03.09. Isolated dog pancreas microsomes after translation were treated with proteases with or without triton X-100 Fluorographs were exposed for 4 days at -70°C.

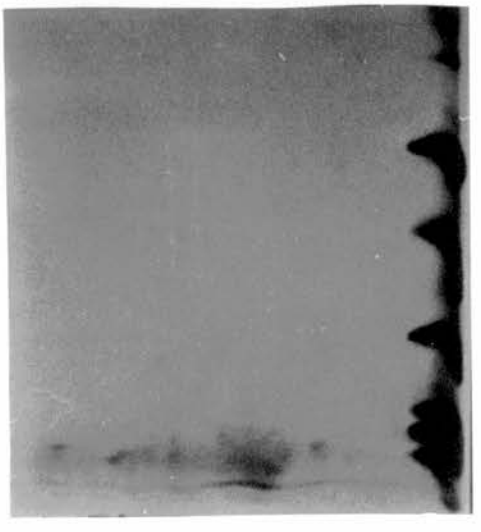
FIGURE 4.06.05.
proteins protected by microsomes from trypsin and chymotrypsin



Control Triton X100 added to microsomes (4.06.05.)

Proteins protected by microsomes from pronase
FIGURE:- 4.06.06.

Control Triton X100 added to microsomes (4.06.06)

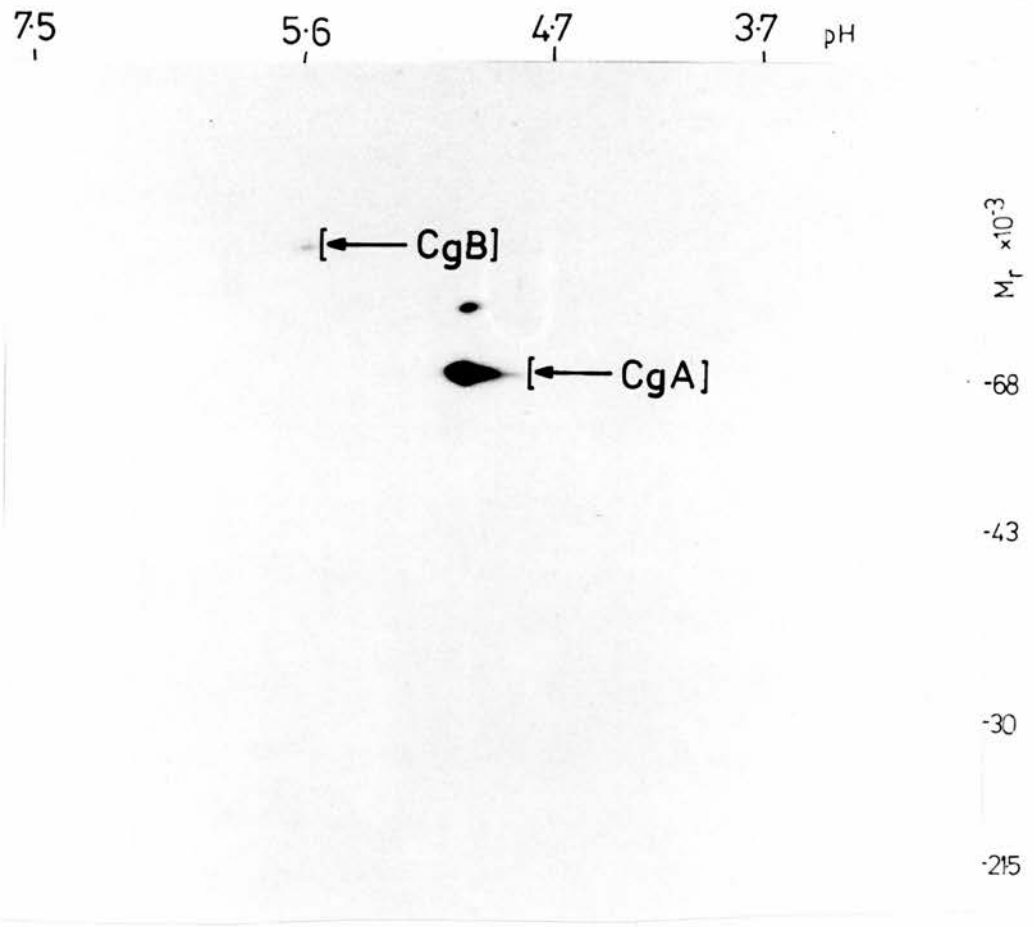


When translations (52µl), performed in the presence of microsomal membranes, were supplemented with chymotrypsin and trypsin (Section 2.03.09.) and the resulting radiolabelled polypeptides were analysed by 2-dimensional gel electrophoresis (Section 2.04.04.) and fluorography (Section 2.04.02.), eight polypeptides were found to be protected from the added proteases by the microsomes (Fig.4.06.05.). However, control experiments, in which microsomal membranes were disrupted by Triton X-100 prior to the addition of trypsin and chymotrypsin, revealed that one polypeptide, of low molecular weight and pI, was resistant to these proteases (Fig.4.06.05.). Consequently a harsher protease, pronase, was used in an attempt to determine whether or not this polypeptide was translocated across the microsomal membranes. The result (Fig.4.06.06.) indicates that this polypeptide could also be degraded, and therefore was not translocated. As discussed in Chapter 7 this polypeptide was subsequently identified as calmodulin.

The fact that several proteins were protected by the microsomes from exogenous proteases, but were destroyed in the control experiments, indicated that the dog pancreas microsomes were functional and also that among the isolated adrenal medullary messenger RNA molecules there were messages encoding secretory or membrane proteins. The two possibilities could be distinguished by separating the microsomal membranes from their contents by treating the membrane vesicles with dilute alkali solution as recommended by Fujiki et al., (1982) (Section 2.03.10.).

FIGURE:- 4.06.07.

Contents of microsomes after alkali wash



Radioactively-labelled polypeptides synthesized during in vitro translation of adrenal medullary messenger RNA in reticulocyte lysate in the presence of dog pancreas microsomes, which were subsequently determined to be located as contents of the microsomal vesicles by alkaline washing as described in 2.03.10. Fluorographs were exposed for 4 days at -70°C.

4.06.03.: Separation of Microsomal Membranes from Microsomal Contents

The washed microsomal membrane vesicles (Section 2.03.08.) from a translation (52µl) of adrenal medullary messenger RNA (Section 2.02.09.) had their contents removed by alkali treatment (Section 2.03.10.). Newly-synthesized polypeptides which had translocated across the microsomes into the lumen were released as contents, whereas polypeptides destined to be intrinsic membrane proteins were retained in the membrane pellet. Results from a 2-dimensional gel separation (Section 2.04.04.) and fluorography (Section 2.04.02.) of these fractions revealed that three of the newly-synthesized polypeptides were translocated across the microsomal membranes into the lumen and could be recovered in the alkali wash fraction (Fig.4.06.07.). No polypeptides were found associated with the microsomal membrane, possibly because not enough radiolabel was incorporated into newly-synthesized membrane proteins to enable them to be detected by fluorography. Similarly, the four polypeptides which appeared to be protected by dog pancreas microsomes, but which were not detected as microsomal contents, may not have had sufficient radiolabel to enable them to be detected by fluorography.

4.07.02.:"Run-off" of Bound Polysomes in the Reticulocyte Lysate System

Adrenal medullary membrane-bound polysomes were run-off in nuclease-treated reticulocyte lysate (Section 2.03.03.). Bound polysomes are efficiently run-off in the reticulocyte lysate system (Walter et al., 1979). Bound polysomes were added to a final concentration of 100 A_{260}units/ml of reaction and the optimal ion concentrations were determined to be 1mM EDTA and 77mM K^+. Mg^{2+} was present in the translation reactions at a concentration of 0.5mM because the polysomes were prepared in a Tris-K^+-Mg^{2+} buffer (Section 2.02.12.). The rate of protein synthesis was constant for 45 minutes (results not shown).

Products from the run-off of bound polysomes were examined by 2-dimensional gel electrophoresis (Section 2.04.04.) and fluorography (Section 2.04.02.). As shown in Figure 4.07.02., a large variety of polypeptides was synthesized. Three of the major polypeptides synthesized on bound polysomes (M_r 100,000, pI 5.6; 70,000, pI 5.2; and 21,000, pI 3.8), were also identifiable from their positions on 2-dimensional gels as major products from the translation of isolated poly A^+ RNA. Similarly, two of these polypeptides, the 100 and 70 kilodalton polypeptides, were identifiable as two of the

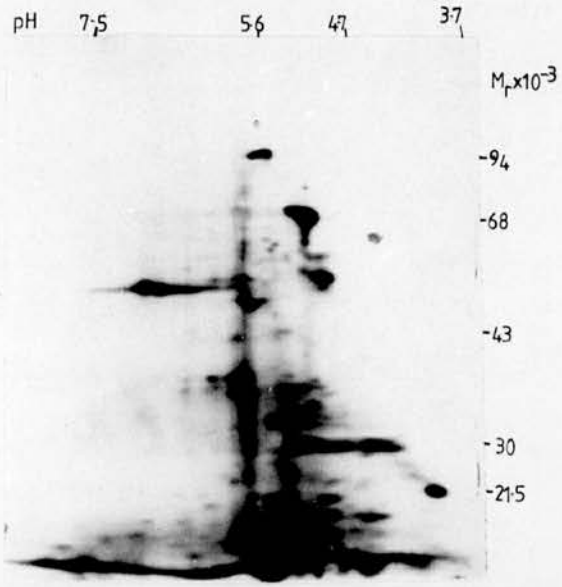


Fig.:-4.07.02. 2-Dimensional gel separation of the polypeptides synthesized when reticulocyte lysate was supplemented with adrenal medullary bound polysomes

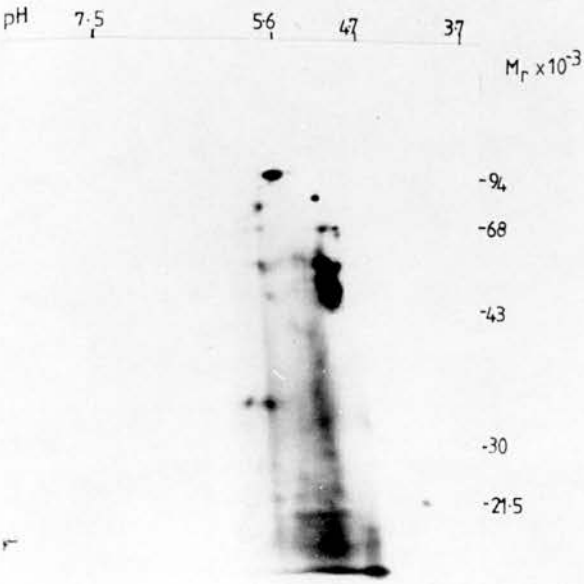


Fig.:-4.07.03. 2-Dimensional gel separation of the polypeptides synthesized when the rat liver-derived P-100 fraction was supplemented with adrenal medullary bound polysomes

Fluorographs were exposed for 4 days @-70°C.

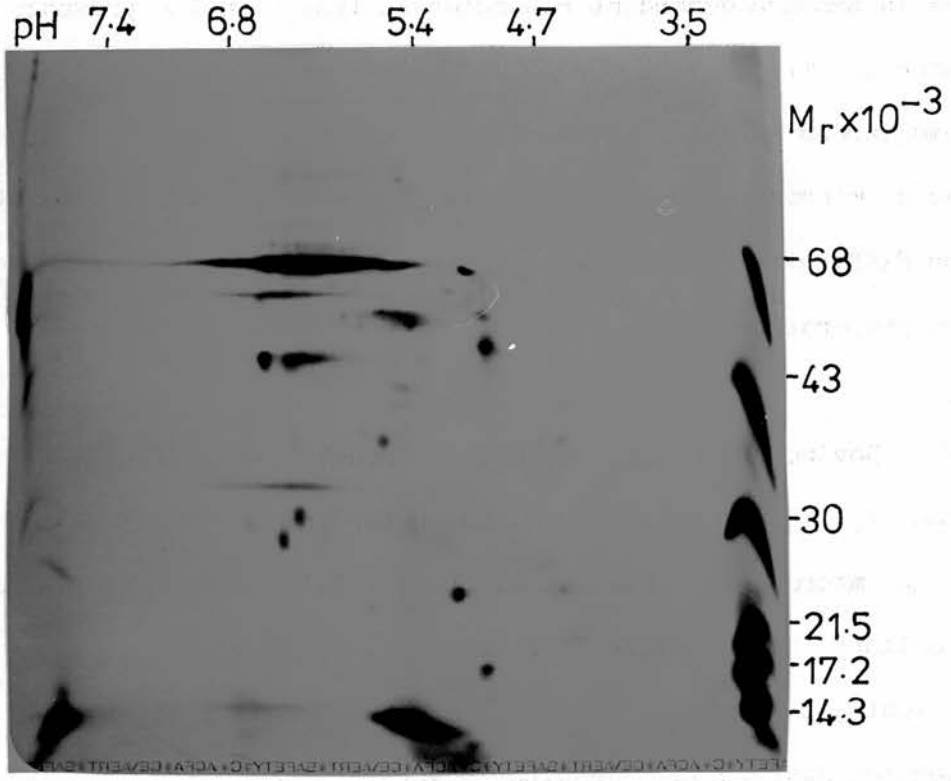
polypeptides which were translocated across dog pancreas microsomes. These polypeptides are in fact the precursors to chromogranin B and chromogranin A respectively (Chapter 5), both major proteins of the chromaffin granule matrix (Sections 1.09.02. and 1.09.03).

4.07.03.: "Run-off" of Adrenal Medullary Bound Polysomes in Rat Liver P-100 Fraction

A Biogel P-100 fraction derived from rat liver high speed supernatant was prepared (Section 2.03.16.) for use as a run-off system for isolated adrenal medullary bound polysomes. It has been reported that this fraction is particularly suitable for polysome run-off because it contains high levels of endogenous ribonuclease inhibitor and polysomes are therefore more stable in this system (Gaetani et al., 1983).

Optimal ion concentrations were determined to be 77mM K^+ and 1mM EDTA. The reactions were performed in the presence of 0.5mM Mg^{2+} from the polysome resuspension buffer (Section 2.02.12.). Polypeptide synthesis was constant for 30 minutes (results not shown). However, despite a high percentage of the ^{35}S-methionine being incorporated into TCA-precipitable material, when examined by 2-dimensional gel electrophoresis and fluorography, the polypeptide products were few, the largest being 70,000 daltons (Fig. 4.07.03.). Presumably the P-100 fraction contained proteases which degraded newly-synthesized polypeptides. Consequently, message-dependent reticulocyte lysate was used as the run-off system for polysome preparations.

FIGURE 4.08.01.: 2-Dimensional gel separation of the polypeptides synthesized when reticulocyte lysate was supplemented with adrenal medullary rough microsomes.



Fluorographs were exposed for 4 days @ -70°C.

4.08.: Adrenal Medullary Rough Microsomes

The isolation of adrenal medullary rough microsomes, that is, a subcellular fraction enriched with ribosome-studded endoplasmic reticulum, was particularly desirable because adrenal medullary nascent polypeptides would be translocated across and processed by adrenal medullary microsomes. Thus, the polypeptide products obtained from in vitro translation of adrenal medullary messenger RNA in message dependent reticulocyte lysate in the presence of dog pancreas microsomes (Section 4.06.01) could be compared with a more homogeneous system. However it is notoriously difficult to prepare rough microsomes that are active in protein synthesis, mainly due to the difficulty of separating rough microsomes from lysosomes during the preparation.

Bovine adrenal medullary rough microsomes were prepared essentially by a method recommended for the preparation of rat liver rough microsomes (Gaetani et al., 1983). Freshly prepared adrenal medullary rough microsomes were added to message dependent reticulocyte lysate to a final concentration of $150A_{260}$ units/ml (Section 2.02.13.). Despite several attempts, there was little success in preparing adrenal medullary rough microsomes which were active in translation. The probable explanation for the failure of this preparation is the presence of contaminating proteases or ribonucleases. The isolation of subcellular fractions from the adrenal medulla is of course complicated by the presence of large quantities of chromaffin granules. However, Fig.4.08.01. shows a

fluorograph of a 2-dimensional gel analysis of the polypeptides synthesized when adrenal medullary rough microsomes were translated by reticulocyte lysate. Some high molecular weight polypeptides were synthesized, although not in large quantities. The 70,000 dalton polypeptide, the predominant product from adrenal medullary messenger RNA translation, can be recognized. Most of the radiolabel however, appears in low-molecular weight species, probably breakdown products.

4.09.: Summary of Translation Systems

Functional messenger RNA can be successfully isolated from any source as long as ribonucleases can be rapidly and efficiently inactivated. Isolation procedures therefore involve the use of strong denaturants such as guanidinium thiocyanate (Chirgwin et al., 1979) or the use of specific ribonuclease inhibitors such as ribonucleoside-vanadyl complexes (Berger et al., 1981). Methods employing ribonuclease inhibitors, usually in combination with phenol, are gentle but often inadequate if the tissue, for example, the spleen, contains high levels of ribonuclease; strong protein denaturants are therefore used to isolate messenger RNA from such tissues.

Bovine adrenal medullary messenger RNA was isolated using guanidinium thiocyanate as denaturant (Chirgwin et al., 1979) in combination with caesium chloride centrifugation (Glisen et al., 1974) as described in Section 2.01.09.. Poly-A^+ RNA was then further purified by affinity chromatography (Aviv and Leder, 1972) as described in Section 2.01.10.. Two cell-free translation systems,

the reticulocyte lysate and the wheatgerm S-30 systems, were optimised for the translation of adrenal medullary messenger RNA and the polypeptide products from each were examined by 1- and 2-dimensional gel electrophoresis. Although both systems were capable of synthesizing a large range of polypeptides, the reticulocyte lysate system was more efficient at synthesizing the largest polypeptides and therefore most subsequent translation experiments were performed with it.

When analysing translation products by 1- or 2- dimensional gel electrophoresis, it is important to remember the limited resolution of fluorography. Some polypeptides will not incorporate sufficient radiolabel to be detectable by fluorography. This may be due to there being only few messenger RNA molecules encoding this protein or the messenger RNA may be a weakly initiating species. The latter possibility invalidates any attempts to correlate the amount of a particular polypeptide synthesized with the amount of messenger RNA encoding it. Alternatively, the polypeptide may have a low content of the radiolabelled amino acid which, for the majority of these studies, was ^{35}S-methionine. Similarly, abundant translation products may be encoded by strongly initiating messenger RNA molecules or they may have a high content of the radiolabelled amino acid. Alternatively there may simply be a large number of messenger RNA molecules encoding the abundant polypeptide. Caution must therefore be adopted when interpreting such results.

The dog pancreas rough microsomes which were prepared were active as judged by their ability to offer protection to translocated

Table 4.01.

A summary of the success of various translation systems when supplemented with adrenal medullary messenger RNA, bound polysomes or rough microsomes

	mRNA	bound polysomes	rough microsomes
Reticulocyte lysate	very good	O.K., but some degradation	degradation a problem
Wheatgerm Extract	O.K., but fewer high M_r products	not done	not done
Rat Liver P-100 #	not done	degradation a problem	not done

polypeptides when challenged with proteases (Section 4.06.03.). The newly-synthesized polypeptides which were protected by the microsomal membranes were subsequently identified as microsomal vesicle contents by alkali treatment of the microsomes (Section 4.06.03.). The polypeptide products from the run-off of adrenal medullary bound polysomes would be expected to be enriched in polypeptides destined for secretion, i.e. chromaffin granule proteins.

A summary of the sytems used to synthesize adrenal medullary polypeptides in vitro and their success is presented in Table 4.01.. Whether any of the polypeptides synthesized from these systems were precursors to the three major chromaffin granule proteins was to be determined by using the antisera described in Chapter 3 to immunoprecipitate their precursors (Chapters 5 and 6). The success of this approach relied on two assumptions: 1. the antisera directed against the chromaffin granule proteins would also recognize the precursors to those proteins 2. enough radiolabel would be incorporated into the immunoprecipitable precursor to allow its identification by fluorography.

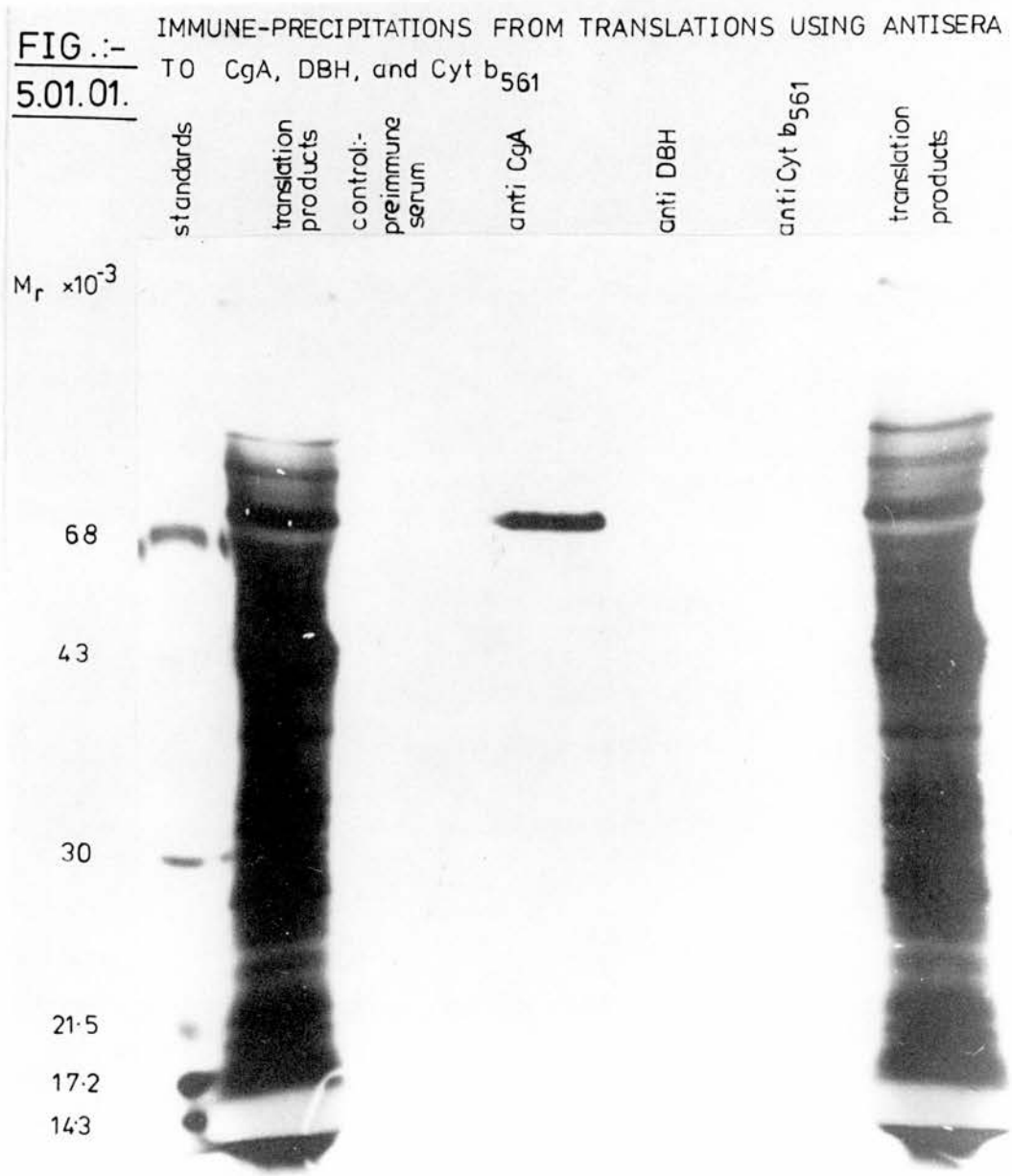
CHAPTER 5

STUDIES WITH CHROMOGRANIN A

RESULTS AND DISCUSSION

FIG.:- 5.01.01.

IMMUNE-PRECIPITATIONS FROM TRANSLATIONS USING ANTISERA TO CgA, DBH, and Cyt b_{561}



Immunoprecipitations were performed as described in 2.03.12. Fluorographs were exposed for **4 days @ -70°C.**

5.01.01.: Immunoprecipitation of the Precursors to Chromaffin Granule Proteins from *in vitro* Translation Reactions

To determine whether any translation products from the *in vitro* translation of adrenal medullary messenger RNA were recognized by antisera to chromogranin A, dopamine β-hydroxylase or cytochrome b_{561}, translation mixes (52μl) containing approximately $6x10^6$ dpm of TCA-precipitable material, performed in reticulocyte lysate as described in Section 2.03.04., were subjected to the procedure for immunoprecipitation using the various antisera as described in Section 2.03.12.. As a control, precipitation using preimmune serum was also performed. The antibody-antigen complexes, eluted from *S. aureus* cells, were prepared for elecrophoresis on an 8-15% SDS-polyacrylamide gel (Section 2.04.01.) and visualized by fluorography. As shown in Fig.5.01.01., the antiserum raised against chromogranin A precipitated a polypeptide from the translation products, which migrated with an apparent molecular weight of 70,000. However, the preimmune serum and the antisera directed against dopamine β-hydroxylase and cytochrome b_{561} did not appear to immunoprecipitate any of the translation products.

5.01.02.:Characterization of the Translation Product Recognized by the Antiserum to Chromogranin A

It was important to ensure that the 70,000 dalton translation product precipitated by the antiserum to chromogranin A was genuinely immunologically related to chromogranin A. This was done by adding chromogranin A, purified by chromatography of the chromaffin granule

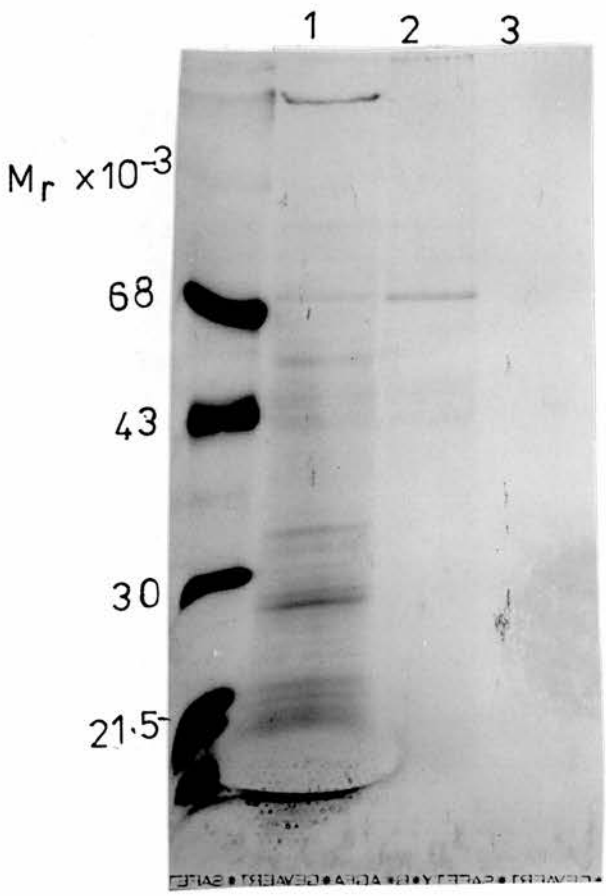
FIGURE 5.01.02.: Competition Experiment :

Purified chromogranin A (20µg) was added to a translation to compete with translation products for immunoglobulins to chromogranin A.

Lane 1 : Total translation products

Lane 3 : Immunoprecipitation of chromogranin A from a translation, performed in the presence of purified chromogranin A

Lane 2 : Immunoprecipitation of chromogranin A from a translation



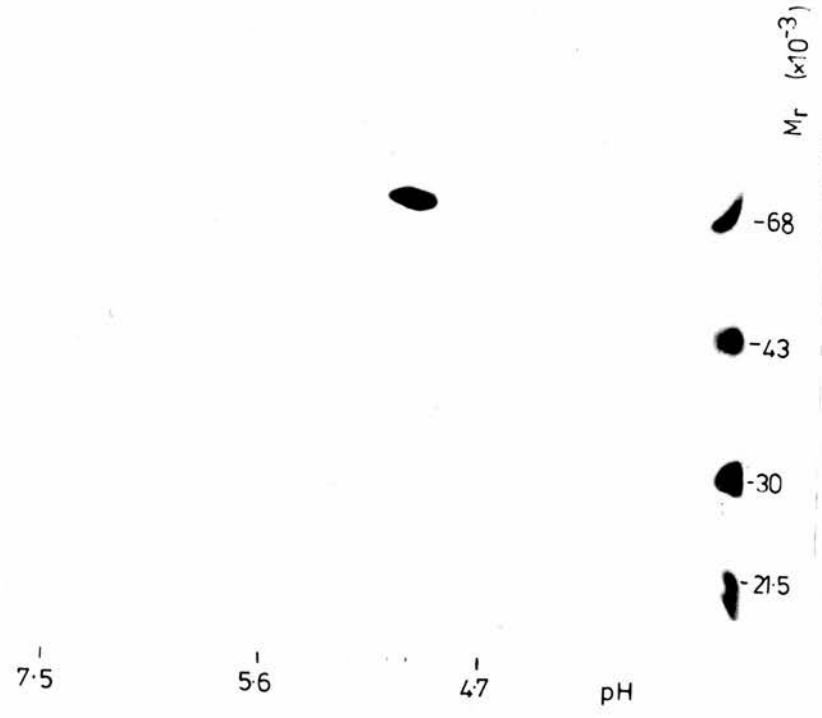
lysate proteins on DEAE-cellulose (Section 2.02.03.) followed by electroelution of chromogranin A from a 10% SDS-polyacrylamide gel, to a translation mix before adding the antiserum against chromogranin A. The purified chromogranin A competed with the 70,000 dalton polypeptide for the immunoglobulins directed against chromogranin A and successfully inhibited the immunoprecipitation of the translation product as shown in Fig.5.01.02.. This suggested that the 70,000 dalton polypeptide was indeed immunologically related to chromogranin A.

More information about this putative precursor to chromogranin A was obtained by 2-dimensional gel electrophoresis (Section 2.04.04.). The fluorograph resulting from 2-dimensional separation of the 70,000 dalton immunoprecipitate revealed that there were approximately equal amounts of two translation products precipitated by the antiserum to chromogranin A (Fig.5.01.04.). These polypeptides apparently differed in molecular weight by

IMMUNE-PRECIPITATION OF CHOMOGRANIN A FROM A

TRANSLATION (2-d separation)

FIG.:- 5.01.04.



Immune Precipitation of CgA from a translation

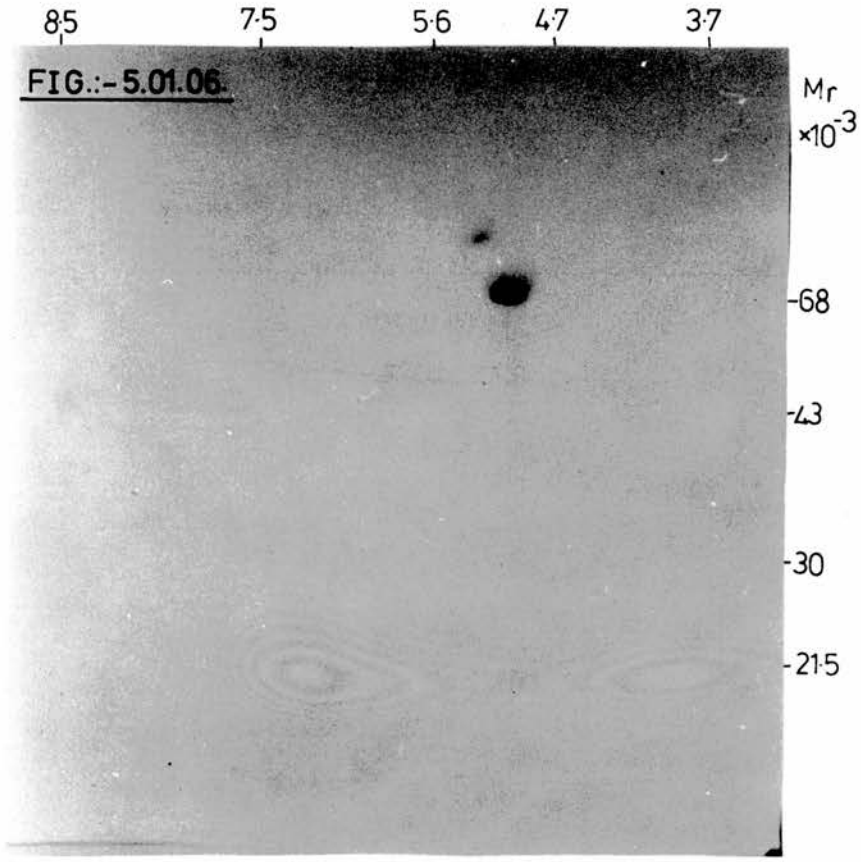
[dotted spot indicates position of Coomassie-stained CgA from lysate which was co-electrophoresed]

FIG.:-5.01.05.

(See 2.03.07. for details of translations). Immunoprecipitations were performed as described in 2.03.12. and fluorographs were exposed for 4 da at -70°C.

500-1000 daltons and had apparent isoelectric points in the region of 5.2, but differing by about 0.05 pH units. The positions of these polypeptides were compared with the position of mature chromogranin A by co-running 50µg of chromaffin granule matrix proteins with the immunoprecipitated translation product on a 2-dimensional gel. The gel was then stained with Coomassie blue before fluorography. The fluorograph could be positioned exactly on top of the Coomassie-stained gel and the position of the immunoprecipitated translation products were then easily compared with the position of mature chromogranin A as shown in Fig.5.01.05.. Preliminary experiments suggested that the primary translation products, recognized by antiserum to chromogranin A, were more acidic than chromogranin A (Kilpatrick et al., 1983a). However, further studies, using a superior method for immunoprecipitations (Anderson and Blobel, 1983) have revealed that the possible putative precursor(s) to chromogranin A are undoubtedly more basic than the mature protein as shown in Fig.5.01.05.. This has been further substantiated by translations performed in the wheatgerm translation system (Falkensammer et al., 1985a) and also by studying the cellular synthesis of chromogranin A (Falkensammer et al., 1985a; Section 5.02.01.). The increased acidity of mature chromogranin A is presumably a result of post-translational O-linked glycosylation of the precursor (Section 1.08.02.), the microheterogeneity of the carbohydrate content being responsible for the heterogeneity of both the molecular weight and pI of chromogranin A. This heterogeneity can be reduced by removing sialic acid residues by neuraminidase treatment (Apps et al., 1985).

CgA immunoprecipitated from a translation in the presence of dog pancreas microsomes



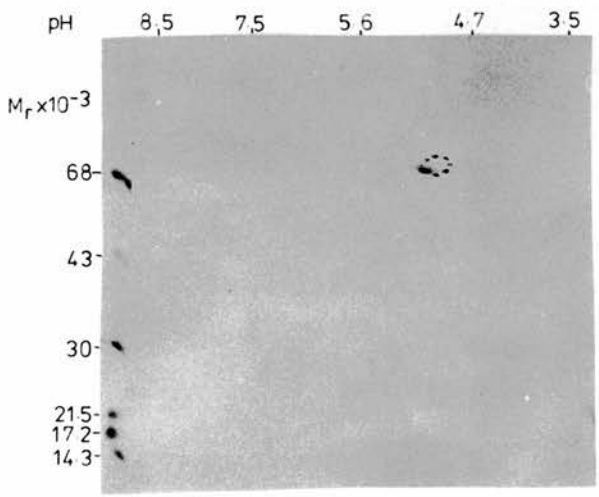


Fig.: 5.01.07. Immunoprecipitation of CgA from a translation in the presence of dog pancreas microsomes. Dotted area indicates Coomassie-stained CgA.

Approximately 4.4% (+/- 0.2%) of the ^{35}S-methionine incorporated into TCA-precipitable material was immunoprecipitated from the translation mix using antiserum against chromogranin A. This was determined by excising and solubilizing the appropriate region of the polyacrylamide gel (Section 2.05.04.) and counting the radioactivity by liquid scintillation counting. This result suggests that a large proportion of the total messenger RNA prepared from the bovine adrenal medulla encodes chromogranin A and/or that the messenger RNA molecule encoding chromogranin A is a strongly initiating species. Chromogranin A contains 13 methionine residues out of a total of 670 amino acids (Smith and Winkler, 1967) and so the polypeptide is not highly labelled due to an unusually large content of the radiolabelled amino acid.

5.01.03.: Cotranslational Processing of the Translation Product Recognized by Antiserum to Chromogranin A

The translation of adrenal medullary messenger RNA was performed in the presence of nuclease-treated dog pancreas rough microsomes (Section 2.03.07.). The microsomes were removed by centrifugation, solubilized and investigated by immunoprecipitation with antiserum to chromogranin A. The resulting eluate from the S. aureus cells was examined by SDS-polyacrylamide gel electrophoresis (Section 2.04.01.) followed by fluorography (Section 2.04.02.). A polypeptide of apparent molecular weight 68,500 was precipitated from the microsomal-associated translation products. A 2-dimensional gel separation of this immunoprecipitate revealed one polypeptide of about 68,500 daltons with an apparent pI of 5.2 (Fig.5.01.06.).

When the position of this polypeptide was compared with that of mature chromogranin A by co-electrophoresis as described in Section 5.01.02., the immunoprecipitate was found positioned at the lowest, most basic region of the mature protein as shown in Fig.5.01.07..

5.01.04.: The Location of the Putative Precursor to Chromogranin A within Microsomal Membrane Vesicles

The 68,500 dalton putative precursor to chromogranin A was protected by the microsomal vesicles from exogenous proteases (Section 2.03.09.) as shown by the ability to immunoprecipitate this polypeptide after treatment of the microsomes with chymotrypsin and trypsin, or pronase. Examination of 2-dimensional gels after co-electrophoresis of the protected polypeptides with 50μg of chromaffin granule matrix proteins showed considerable similarity between the two sets of proteins. To determine the intravesicular location of these newly-synthesized proteins, the microsomal vesicles from a translation of adrenal medullary messenger RNA were washed with sodium carbonate (Section 2.03.10.) in order to separate the microsomal membranes from the soluble contents. The putative precursor to chromogranin A was immunoprecipitable from the microsomal contents but not from the microsomal membranes suggesting that this polypeptide was completely translocated into the lumen of the microsomes. This is consistent with chromogranin A existing only as a soluble protein (Winkler, 1977) and also with its distribution after phase separation of chromaffin granule membranes in Triton X-114 (Pryde and Phillips, 1985). The recent report suggesting that chromogranin A is a major chromaffin granule membrane protein

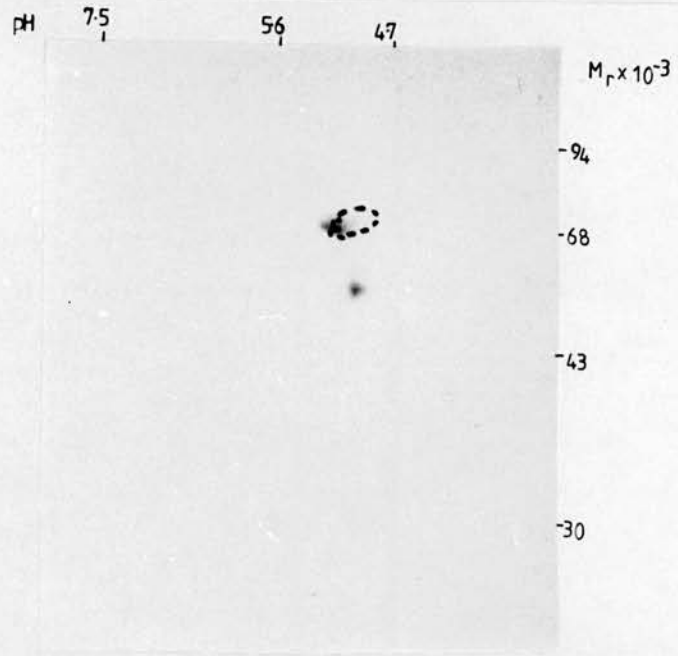


Fig.:- 5.01.08. Immunoprecipitation of polypeptides recognized by antiserum to CgA when bound polysomes were run-off in reticulocyte lysate

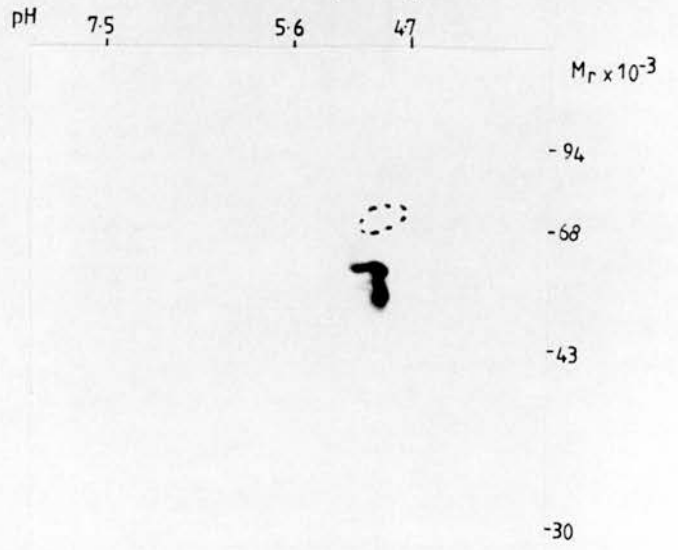


Fig.:- 5.01.09. Immunoprecipitation of polypeptides recognized by antiserum to CgA when bound polysomes were run-off in P-100 fraction, derived from rat liver

Experimental details of the run-off of bound polysomes in reticulocyte lysate and rat liver P-100 fraction is described in Sections 4.07.02 and 4.07.03. respectively. Immunoprecipitations were performed as described in Section 2.03.12.

(Settleman et al., 1985a) is probably a result of contamination of chromaffin granule membranes with chromaffin granule content proteins, due to insufficiently effective washing.

5.01.05.: Immunoprecipitation of the Putative Precursor to Chromogranin A, from the Polypeptide Products obtained by Running-off Bound Polysomes in Reticulocyte Lysate

Adrenal medullary bound polysomes were run-off in message-dependent reticulocyte lysate as described in Section 4.07.02.. Immunoprecipitation using antiserum to chromogranin A was then performed, to determine whether any of the resulting polypeptides were immunologically related to chromogranin A. As shown in Fig.5.01.08., immunoglobulins directed against chromogranin A, immunoprecipitated three polypeptides (two of about 70,000 and one of 53,000 daltons) from the products of run-off of adrenal medullary bound polysomes. When the positions of these polypeptides were compared with those of mature chromogranins A by co-electrophoresing 50μg of chromaffin granule matrix proteins on 2-dimensional gels, the two similar 70,000 dalton polypeptides were located at the most basic region of the chromogranin A spot (Fig.5.01.08.). These polypeptides were in fact located in an identical position to the putative chromogranin A precursors, which had been immunoprecipitated after translations of adrenal medullary messenger RNA in the absence of microsomes. The 53,000 dalton polypeptide precipitated from the bound polysomal run-off products was probably derived from chromogranin A by the action of residual

protease activity in the polysome preparation. It co-migrated with a member of the chromogranin A family (chromogranin A_3; see Fig. 5.03.02.) as determined by comparing a Coomassie-stained gel with the fluorograph.

5.01.06.: Immunoprecipitation of the Putative Precursor to Chromogranin A from the Polypeptide Products obtained by Running-off Adrenal Medullary Bound Polysomes in a Biogel P-100 Fraction from Rat Liver

Adrenal medullary bound polysomes were run-off in a Biogel P-100 fraction derived from rat liver high speed supernatant (Section 4.07.03.). An immunoprecipitation using antiserum to chromogranin A was performed (Section 2.03.12.) to determine whether any of the resulting polypeptides were immunologically related to chromogranin A. Two-dimensional gel electrophoresis and fluorography of the immunoprecipitate revealed that several polypeptides, synthesized by the run-off of adrenal medullary bound polysomes and migrating with apparent molecular weights of between 50,000 and 60,000 daltons, were immunologically related to chromogranin A (Fig.5.01.09). These polypeptides co-migrated with members of the chromogranin A family and probably resulted from contaminating proteases acting on the 70,000 dalton polypeptide product synthesized when bound polysomes were run-off in reticulocyte lysate. As mentioned previously (Section 4.07.03.), contaminating protease activity was not such a problem when running-off bound polysomes in reticulocyte lysate. This may be due to the lack of protease inhibitors in the Biogel P-100 fraction or to the presence of

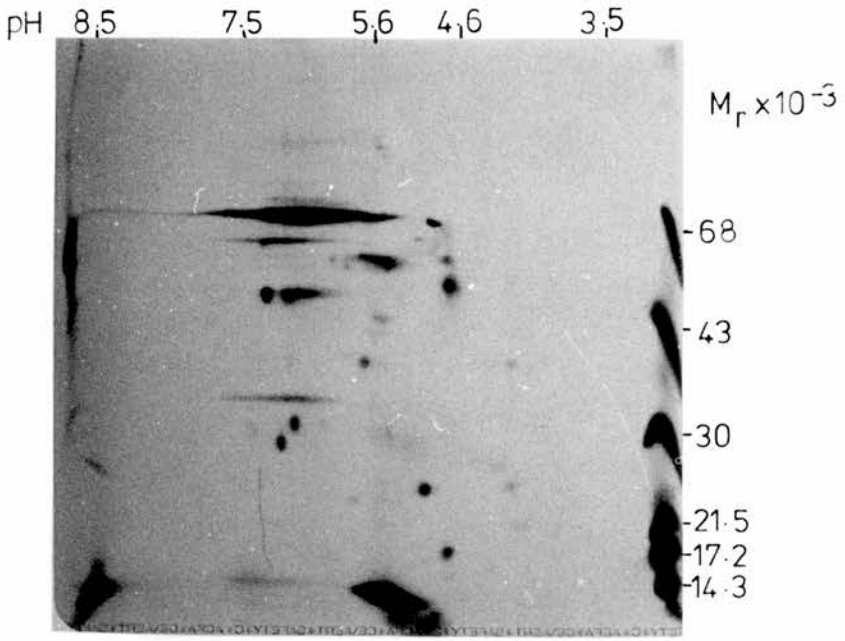


Fig.: 5.01.10. Polypeptides synthesized when reticulocyte lysate was supplemented with rough microsomes.

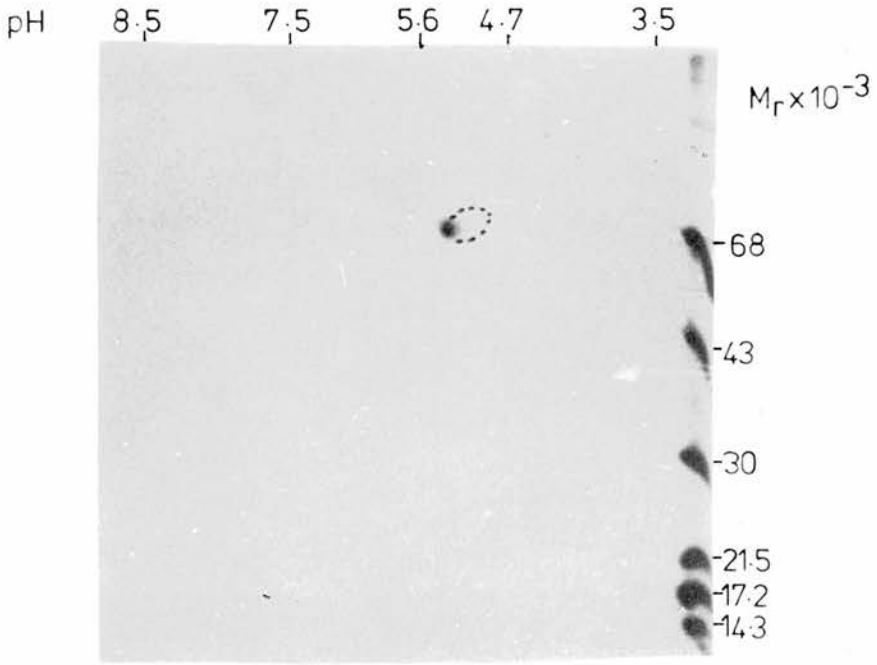


Fig.: 5.01.11. Immunoprecipitation of CgA from the polypeptide products when reticulocyte lysate was supplemented with rough microsomes. Dotted area indicates Coomassie-stained CgA.

proteases in this fraction. Reticulocyte lysate is known to contain endogenous protease inhibitors (Hunt and Jackson, 1974).

5.01.07.: Identification of Chromogranin A among the Polypeptides Synthesized by Rough Microsomes

As discussed in Section 4.08., attempts to prepare adrenal medullary rough microsomes which were active in protein synthesis were largely unsuccessful. However, radiolabelled polypeptide products were obtained from several preparations of rough microsomes as shown, for example, in Fig.5.01.10. It was possible to immunoprecipitate a polypeptide with antiserum to chromogranin A from the polypeptides synthesized from adrenal medullary rough microsomes. When examined by 2-dimensional gel electrophoresis, this polypeptide co-migrated with the lowest molecular weight, most basic region of the chromogranin A spot (Fig.5.01.11.). It ran in an identical position to the polypeptide immunoprecipitated from a translation of adrenal medullary messenger RNA performed in the presence of dog pancreas rough microsomes (Fig.5.01.12.).

5.01.08.:The Biosynthesis of Chromogranin A <u>in vitro</u>

The above results suggest that there may be two primary precursors to chromogranin A (Section 5.01.02.) which are synthesized on polysomes bound to the endoplasmic reticulum (Section 5.01.05.). The precursors have similar apparent molecular weights and isoelectric points of about 70,000 and 5.2 respectively, but can be clearly resolved by 2-dimensional gel electrophoresis. Both precursors are

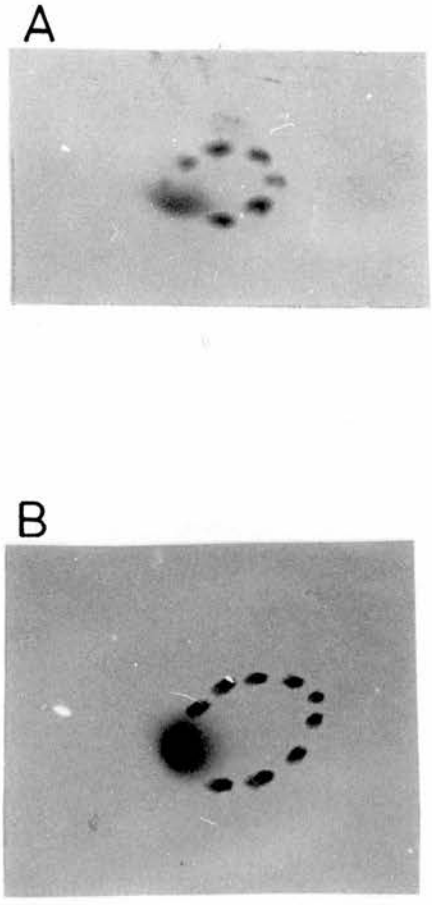


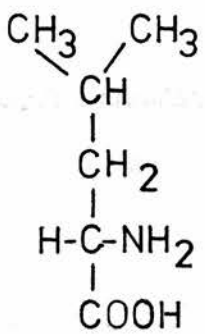
Fig.: 5.01.12. Immunoprecipitation of CgA from the polypeptides synthesized when reticulocyte lysate was supplemented with A. dog pancreas microsomes and mRNA
B. rough microsomes

more basic than mature chromogranin A (Fig.5.01.05.). However, only one polypeptide, which is protected by microsomes from exogenously added proteases, is immunoprecipitated from translations of adrenal medullary messenger RNA, performed in the presence of dog pancreas rough microsomes (Section 5.01.03.) or from the polypeptides synthesized from adrenal medullary rough microsomes (Section 5.01.07.). This polypeptide is translocated across the microsomal membrane into the lumen as shown by its release by sodium carbonate treatment of microsomal vesicles.

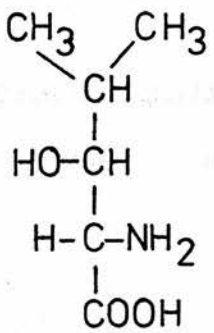
There are three possibilities which could account for the existence of two precursors to chromogranin A. They could simply be the products of two different genes. Alternatively, one gene could produce two different messenger RNA's by different splicing events. This phenomenon has already been discussed in Section 1.03.01.. Yeast cells use this mechanism to produce cytoplasmic and secretory invertase (Perlman et al., 1982) as do lymphocytes to produce membrane bound and secretory forms of immunoglobulin M (Early et al., 1980). Why would an adrenal medullary cell produce two messenger RNA molecules encoding its major secretory protein? Presumably this would be of some physiological significance but while the function of chromogranin A remains unknown, the reason for having two precursors will also remain unknown.

The third possibility is that the two putative precursors to chromogranin A differ only in their signal sequences. The initiation of protein synthesis usually occurs at the first AUG triplet from the 5' end of the messenger RNA. However, there are exceptions to this

Fig.: 5.01.13.



L-leucine



L-β-hydroxyleucine

rule (Kozak, 1983). Initiation occurs at the first and second AUG triplets for example, in an immunoglobulin messenger RNA (Kelley et al., 1982). Also, parathyroid secretory protein I which, as discussed in Section 1.09.04., is apparently identical to chromogranin A, is thought to have four precursors differing only in their signal sequences (Majzoub et al., 1979; 1982).

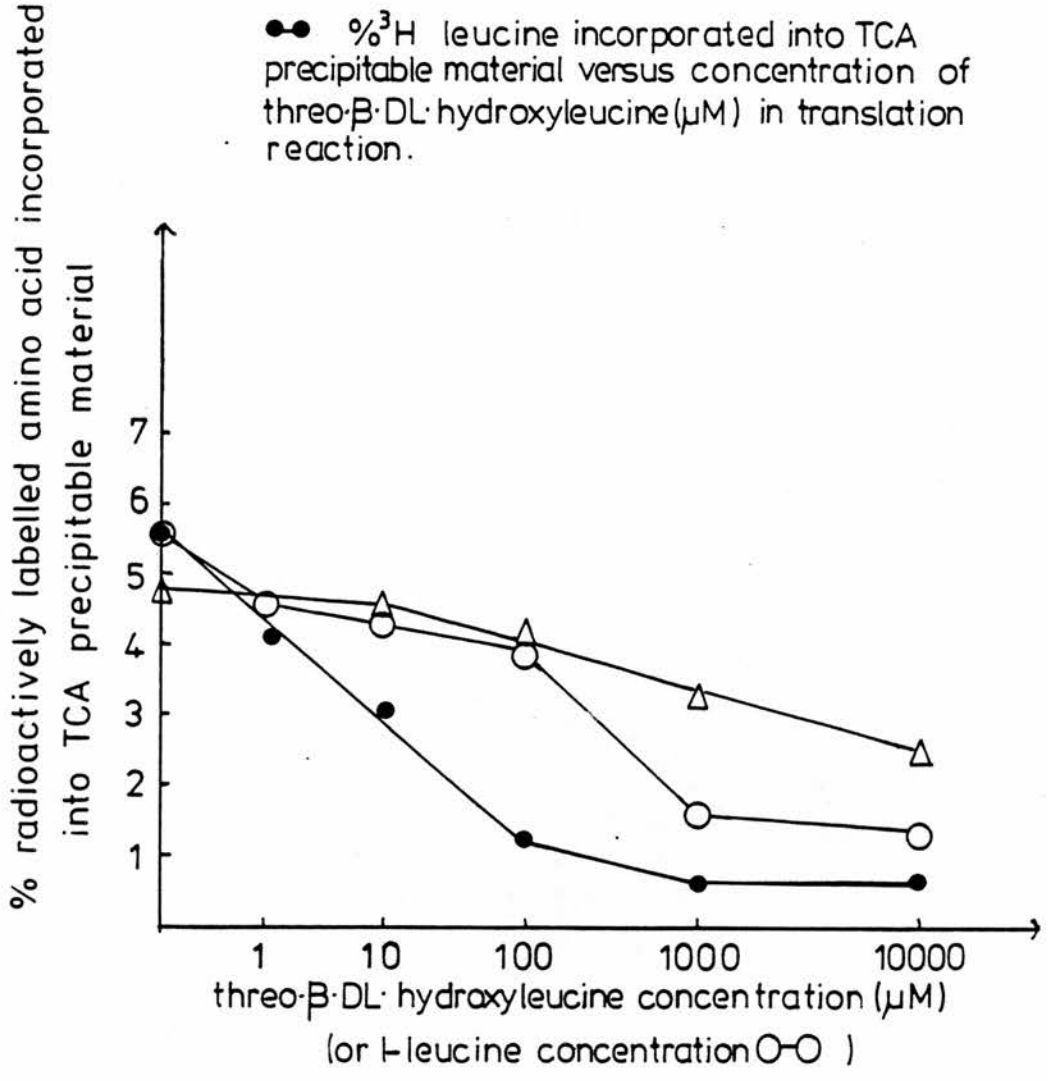
5.01.09.:An Attempt to Distinguish between the Putative Precursors to Chromogranin A using the Amino Acid Analogue threo-β-DL-Hydroxyleucine

An attempt was made to determine whether the two precursors to chromogranin A had signal sequences which differed in their leucine content. All secretory protein signal sequences are characterized by a central hydrophobic region which is often rich in leucine. The integrity of a signal sequence with a high content of leucine can be upset by incorporating the amino acid analogue, threo-β-DL-hydroxyleucine (Fig.5.01.13.), during protein synthesis with the result that the polypeptide is not translocated across the microsomal membrane nor is the signal sequence cleaved (Hortin and Boime, 1980a; 1980b; 1983). This approach was used in the present study of chromogranin A synthesis. It was expected that, when synthesized in the presence of β-hydroxyleucine, a polypeptide with a signal sequence normally rich in leucine would not be translocated across the microsomal membrane and would not have its signal sequence cleaved, whereas a polypeptide with a low content of leucine residues might be translocated and proteolytically processed as usual.

Amino acid analogues are incorporated into protein less

Fig.:- 5.01.14. Δ–Δ $\%^{35}S$ methionine incorporated into TCA precipitable material versus concentration of threo·β·DL· hydroxyleucine (μM) in translation reaction.

O–O $\%^{3}H$-leucine incorporated into TCA precipitable material versus concentration of L-leucine (uM) in translation reaction



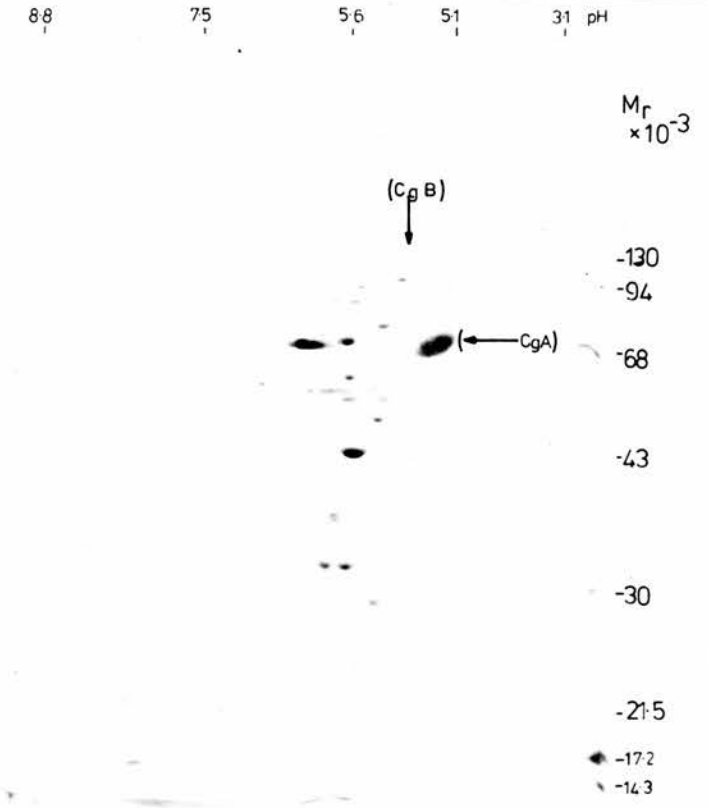
[All translation reactions were performed in message dependent reticulocyte lysate using adrenal medullary mRNA and were incubated for 60minutes at 30°C.]

efficiently than natural amino acids and therefore the analogue must be present in excess during the translation reaction. The optimal concentration of threo-β-DL-hydroxyleucine was determined by examining the effect of varying concentrations of threo-β-DL-hydroxyleucine on the incorporation of ^{3}H-leucine and ^{35}S-methionine into TCA-precipitable material (Fig. 5.01.14). The inhibitory effect of threo-β-DL-hydroxyleucine on the incorporation of ^{3}H-leucine into TCA-precipitable material was compared with that of L-leucine (Fig.5.01.14). At a concentration of 20-30μM, L-leucine inhibited the incorporation of ^{3}H-leucine by 50% whereas, this degree of inhibition was achieved by threo- -DL-hydroxyleucine at 600μM. When 10mM threo-β-DL- hydroxyleucine was present in the translation reaction, 80% inhibition of the ^{3}H-leucine incorporation was achieved. As shown in Fig. 5.01.14., 10mM threo-β-DL-hydroxyleucine inhibited the incorporation of ^{35}S-methionine by about 50%.

Subsequently, translations in the presence of dog pancreas microsomes were performed in the presence of 10mM threo-β-DL-hydroxyleucine. Immunoprecipitations using antiserum to chromogranin A were performed from the solubilized microsomal pellet (Section 2.03.08.) containing the processed polypeptides and from the remaining supernatant, containing the unprocessed polypeptides. The resulting fluorographs of the immunoprecipitations did not indicate that the processing of one of the putative precursors to chromogranin A was more sensitive to the presence of threo-β-DL-hydroxyleucine than the other. In fact, both precursors appeared rather insensitive to β-hydroxyleucine, in as much as they were both translocated and proteolytically processed as normal.

FIG.:- 5.02.01

Cells: Coomassie Blue Stained Gel of cellular proteins



Cell isolation : 5 minute pulse with ^{35}S-methionine

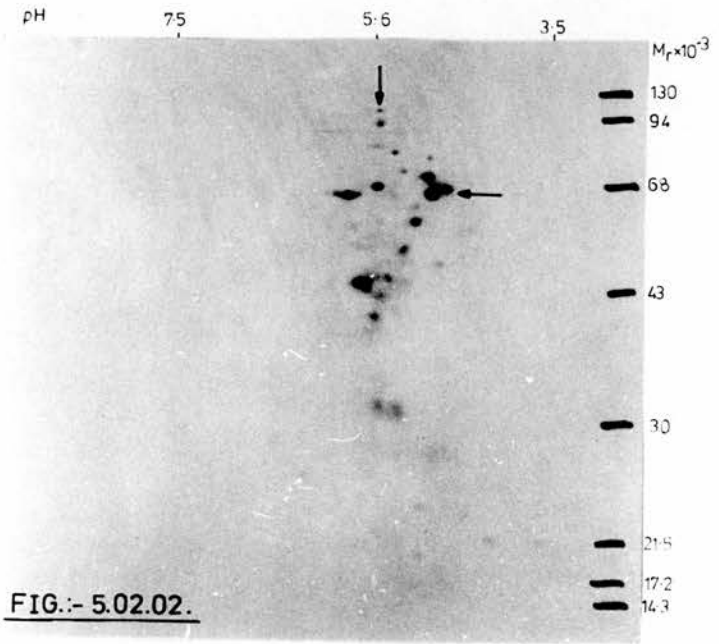


FIG.:- 5.02.02.

Experimental details can be found in 2.02.14. of adrenal medullary cell isolation and labelling of newly-synthesized proteins with ^{35}S-methioni Fluorographs were exposed for 4 days @ -70°C.

These results therefore suggest that both putative precursors to chromogranin A have signal sequences with a low leucine content. This does not of course, rule out the possibility that the signal sequences differ in other respects.

5.02.01.: Cellular Synthesis of Chromogranin A

The results discussed previously using in vitro translation systems (Section 5.01.) have given a good indication of the initial events involved in the biosynthesis of chromogranin A. Further information about the post-translational processing of chromogranin A was obtained by radiolabelling the newly-synthesized proteins of isolated adrenal medullary cells.

Bovine adrenal medullary cells were isolated as described in Section 2.02.14.. The cells were then incubated with ^{35}S-methionine for 5, 15, 30 or 60 minutes. Alternatively, the cells were pulse-labelled with ^{35}S-methionine for 5 minutes, then chased with excess "cold" methionine for 5, 15 or 45 minutes. Proteins from 1.5×10^6 were examined by 2-dimensional gel electrophoresis (Section 2.04.04.) and staining (Section 2.04.01.) and fluorography (Section 2.04.02.). It was not possible to immunoprecipitate the newly-synthesized, radiolabelled polypeptides, possibly due to the large amounts of unlabelled proteins present in the cells before the addition of the ^{35}S-methionine. The alternative, although not ideal technique, was to overlay the fluorograph on the stained gel. The chromogranin A spot, for example, is of characteristic shape,

Cell isolation : 30minute pulse with ^{35}S-methionine

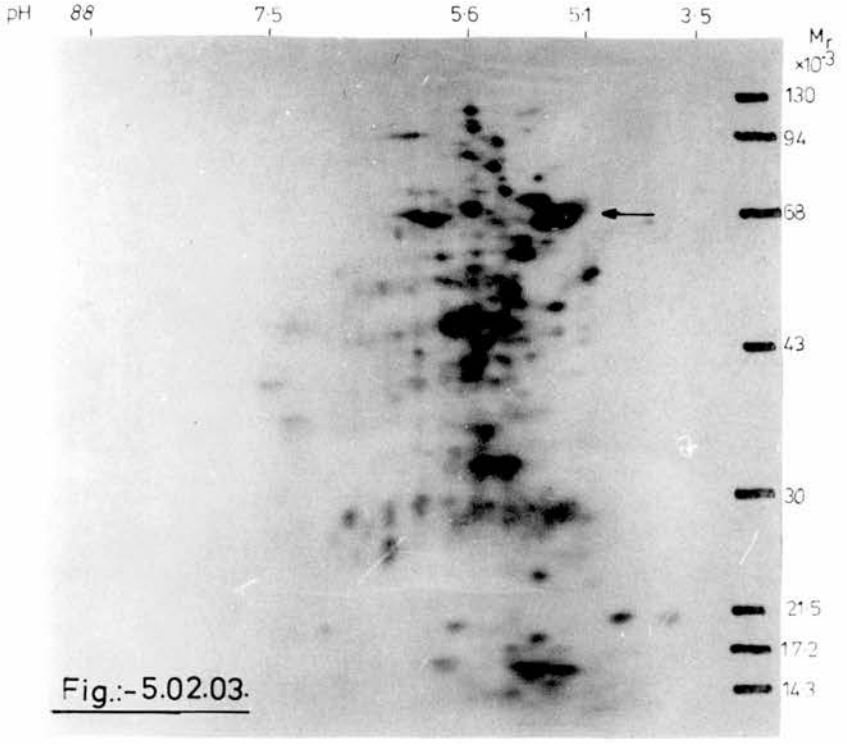


Fig.:-5.02.03.

Cell isolation : 5minute pulse with ^{35}S- methionine followed by a 15minute chase with cold methionine

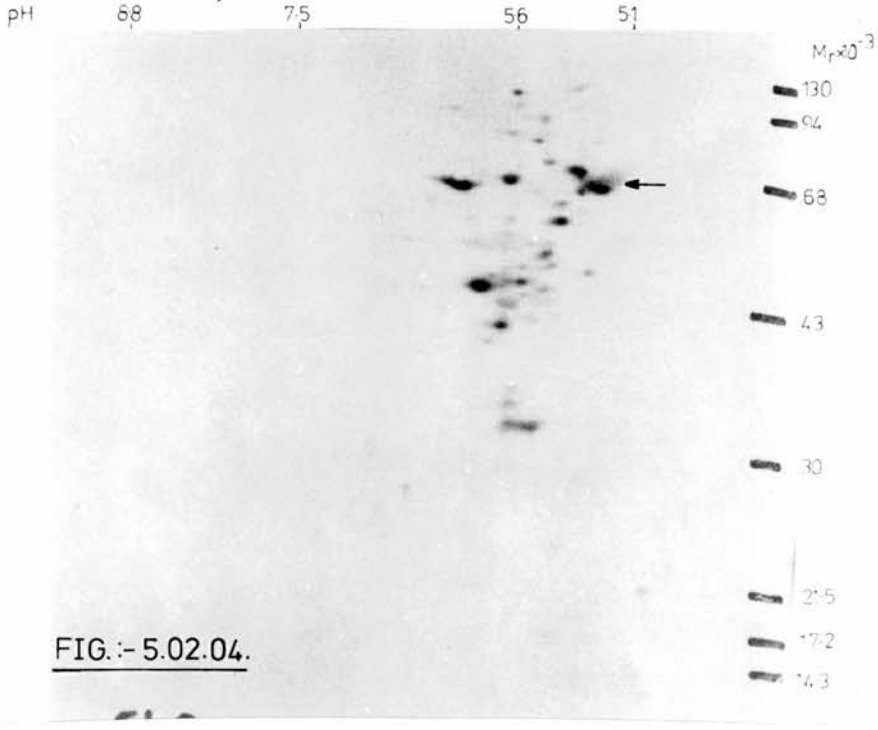


FIG.:-5.02.04.

Experimental details can be found in 2.02.14. of adrenal medullary cell isolation and labelling of newly-synthesized proteins with ^{35}S-methionine Fluorographs were exposed for 4 days @ -70°C.

molecular weight and isoelectric point, and is easily identifiable on 2-dimensional Coomassie-stained gels (Fig.5.02.01.). By this method, the change in the shape and position of the newly-synthesized chromogranin A spot was followed through the different stages of its maturation. After a 5 minute pulse with ^{35}S-methionine, the newly-synthesized chromogranin A was located in the lowest molecular weight, most basic region of the large Coomassie-stained chromogranin A spot (Fig.5.02.02). This position is similar to that of the polypeptide immunoprecipitated from *in vitro* translation of adrenal medullary messenger RNA, performed in the presence of dog pancreas microsomes (Fig.5.01.06). The two primary precursors to chromogranin A, synthesized from the translation of messenger RNA in reticulocyte lysate (Fig.5.01.04) would presumably be cotranslationally processed and therefore undetectable in the cellular protein synthesis. When the isolated cells were labelled with ^{35}S-methionine for 15 minutes, the newly synthesized chromogranin A spot was completely superimposible on that of Coomassie-stained chromogranin A (Fig. 5.02.03), indicating that the newly synthesized chromogranin A had undergone post-translational processing, which in the case of chromogranin A is O-linked glycosylation (Kiang *et al.*, 1982; Fischer-Colbrie *et al.*, 1982; Section 1.09.02.), sulphation (Falkensammer *et al.*, 1985b), and phosphorylation (Settleman *et al.*, 1985b). Longer incubations with radiolabel resulted in more radiolabel being incorporated into protein but did not reveal any further shifts in the chromogranin A spot.

The pulse-labelling experiments revealed the same processing

Section of gel of microsomal pellet showing shifts occurring due to post translational modification of CgA (O-glyc-osylation) and of CgB (sulph-ation)

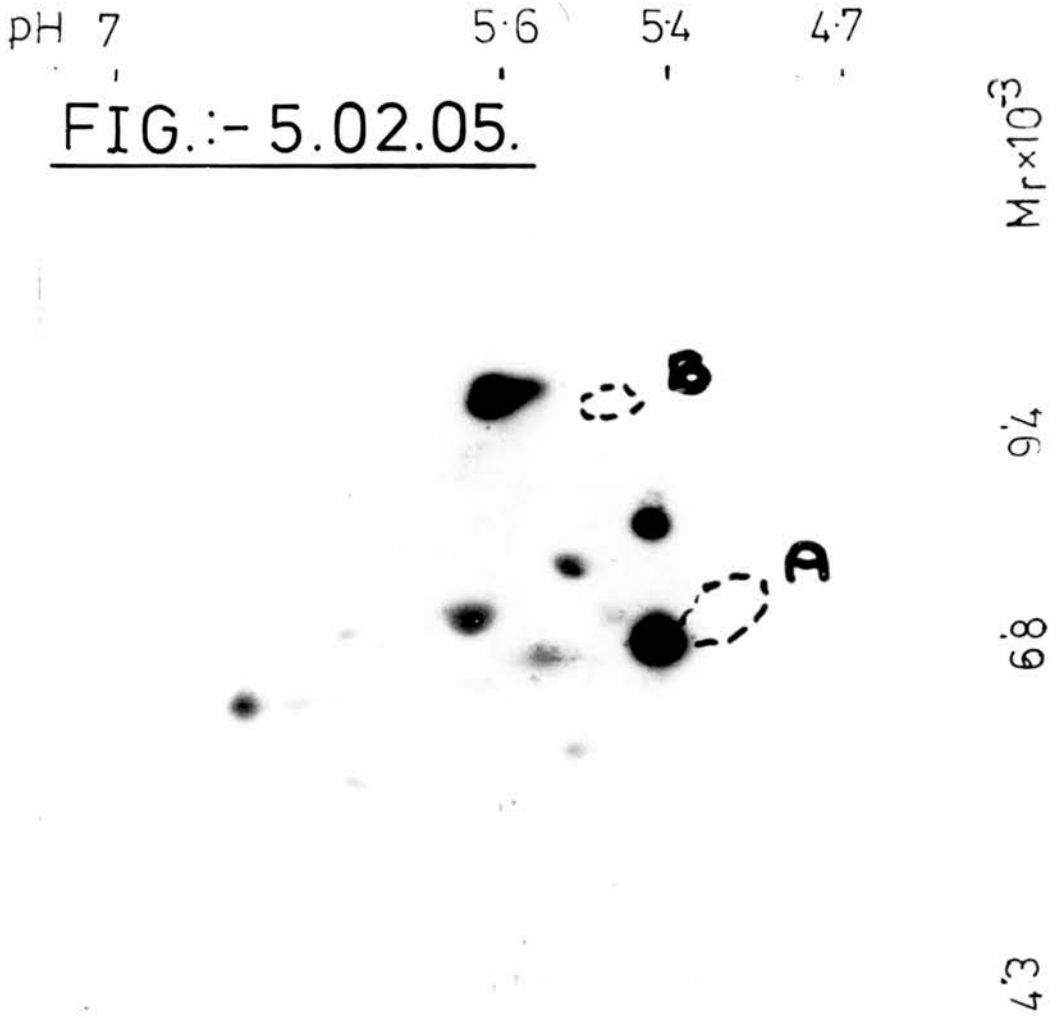
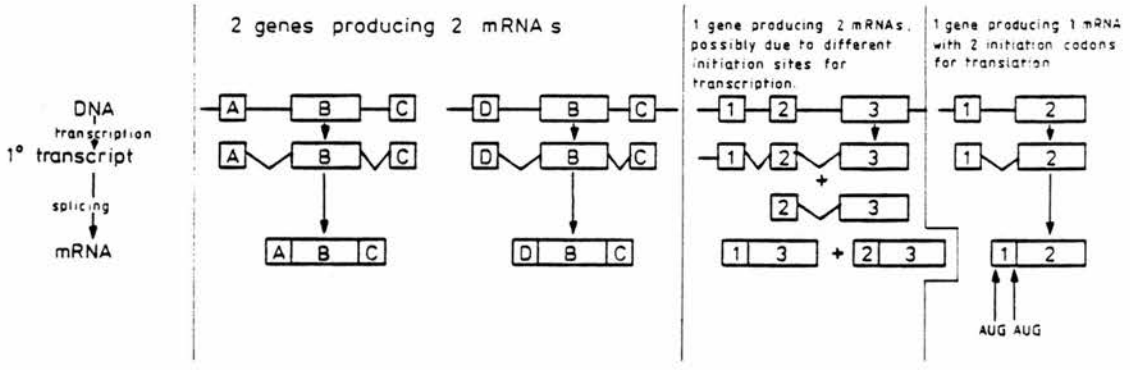


FIG.:- 5.02.05.

events in a different way. After incubating the isolated cells with ^{35}S-methionine for 5 minutes, the newly-synthesized chromogranin A was positioned at the lowest, most basic region of the Coomassie-stained chromogranin A spot. However, chasing the cells with "cold" methionine prevented further incorporation of radiolabel into protein. Thus, the fluorographs of the proteins from cells which had been pulse-labelled and then chased for various lengths of time revealed a shift of the radiolabelled chromogranin A towards increased molecular weight and acidity. This is presumably due to the addition of O-linked sialic acid-containing carbohydrate chains to the chromogranin A synthesized during the initial 5 minute pulse (Fig.5.02.04.), and to the attachment of other acidic groups, such as phosphate and sulphate. The fluorographs from the pulse-chase experiments also demonstrate nicely the isoelectric point shift in chromogranin B during post-translational processing (Fig.5.02.05.). Sulphation, a post-translational processing event occurring in the trans Golgi region, is responsible for the shift of chromogranin B from a pI of 5.6 to a pI of 5.4 (Falkensammer et al., 1985b).

It is interesting that none of the lower molecular weight members of the chromogranin A or chromogranin B families were radioactively labelled, even after 60 minutes, although they were present in the Coomassie-stained gels of the cellular proteins. In fact, these proteins were not even found to be radioactively labelled after an 18 hour incubation (J.G. Pryde, personal communication) suggesting that proteolytic processing of chromogranin A, from its 70,000 dalton form to smaller peptides, is very slow.



Either 1 or 2 mRNAs encoding chromogranin A

Cotranslational segregation into the E.R lumen accompanied by signal sequence cleavage.

1 polypeptide $M_r \sim 68{,}000$

pI ~ 5·4

Rapid transport through the Golgi (5-15 min.).

O-linked glycosylation; tri- and tetra-saccharides composed of GalNAc, Gal and NANA, thereby increasing the M_r and acidity, leading to microheterogeneity.

Packaging of chromogranin A within immature chromaffin granules.

After 24 hours or longer, about 50% of the chromogranin A is proteolytically digested, giving rise to the smaller members of the chromogranin A family.

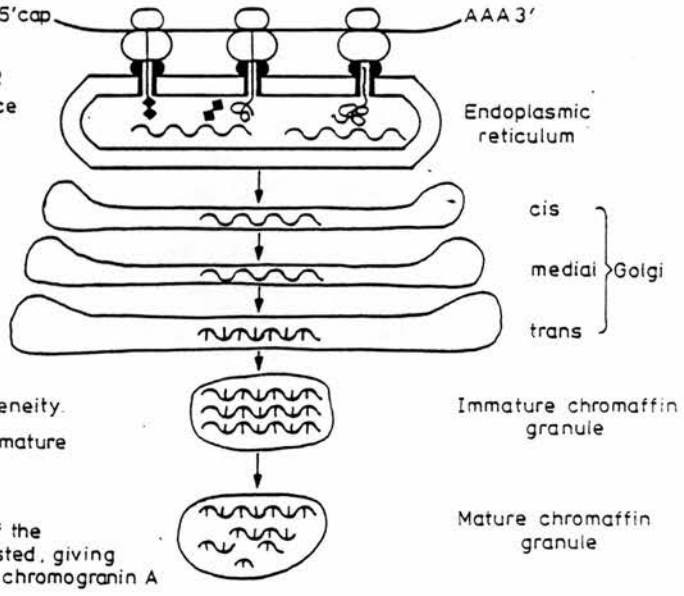


Fig. 5.02.06. A diagrammatic summary of the biosynthesis of chromogranin A.

5.02.02. The Biosynthesis of Chromogranin A

The processes thought to occur during the biosynthesis of chromogranin A, combining the results from the in vitro translation studies and cellular synthesis, have been summarized in Fig. 5.02.06.. The origin of the two primary polypeptide precursors (Section 5.01.02) is likely to remain a mystery until the gene(s) encoding chromogranin A have been cloned and sequenced. However, post-translational processing of the initial translation product, a polypeptide of apparent molecular weight and isoelectric point 68,500 and 5.2 respectively, can be correlated with the carbohydrate content of chromogranin A.

However, the two translation products could arise from a single translation product by a minor modification such as fatty acylation or proteolysis.

As mentioned previously (Section 1.09.02.), carbohydrate analysis of chromogranin A suggests that it contains O-linked tri- and tetra- saccharides composed of N-acetyl galactosamine, galactose and N-acetyl and N-glycolyl neuraminic acids (Kiang et al., 1982; Fischer-Colbrie et al., 1982). O-linked glycosylation is initiated in the medial Golgi region and completed in the trans Golgi region by galactosyl and sialyl transferases. Chemical treatment of chromogranin A to remove the carbohydrate residues, increases its isoelectric point by up to 0.4 pH units (Apps et al., 1985), although neuraminidase treatment produced rather smaller shifts. Chromogranin A contains 6 moles of neuraminic acid per mole of protein (Kiang et al., 1982; Fischer-Colbrie et al., 1982). The removal of one neuraminic acid residue from glycoprotein III increases the isoelectric point by 0.08 pH unit (Gavine et al., 1984) and therefore the change in the isoelectric point of chromogranin A corresponds quite nicely to the removal of all the neuraminic acid

residues (Apps et al., 1985). Conversely, addition of carbohydrate to the chromogranin A precursor would increase its molecular weight and decrease its isoelectric point. The heterogeneity of the isoelectric point of chromogranin A is probably due to the microheterogeneity of the carbohydrate chains. Chemical deglycosylation reduces the heterogeneity of the isoelectric point of the chromogranin A spot on SDS-polyacrylamide gels but does not have such an obvious effect on the heterogeneity of the molecular weight (Apps et al., 1985). The shift of radiolabelled chromogranin A, synthesized by isolated cells during a 5 minute pulse with ^{35}S-methionine, towards increased molecular weight and acidity after a 15 minute chase with cold methionine (Fig. 5.02.04), is most likely due to the O-glycosylation of the 68,500 dalton precursor polypeptide.

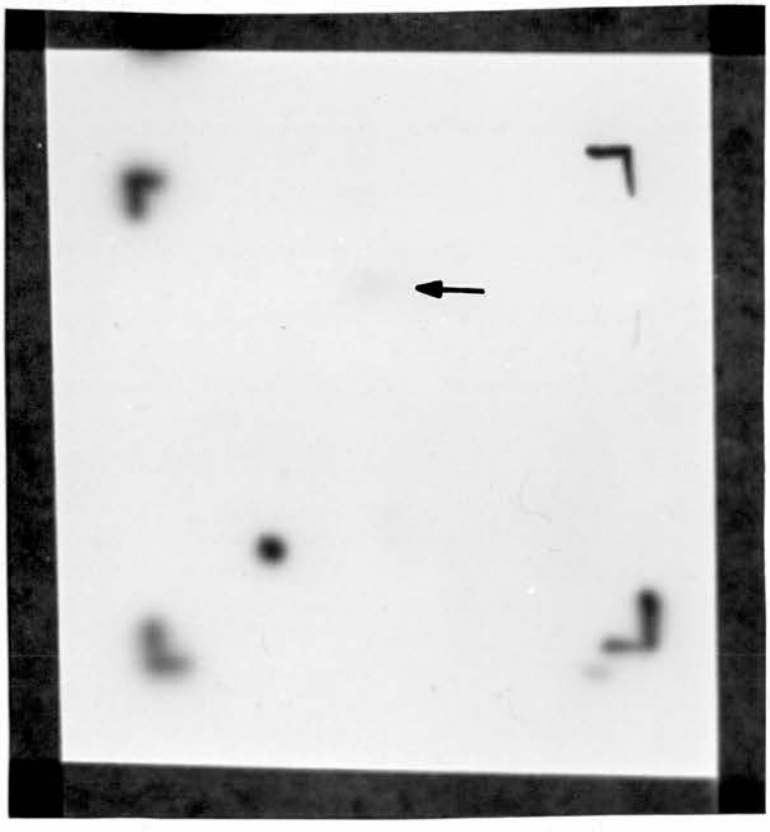
These studies support Winkler's suggestion that the smaller chromogranins arise as a result of intragranular protease action on chromogranin A (Winkler, 1976). Only two polypeptides of very similar molecular weight and isoelectric point (Fig.5.01.04) were immunoprecipitated by anti-chromogranin A serum from the products obtained when adrenal medullary messenger RNA was translated in reticulocyte lysate (Section 5.01.; Kilpatrick et al., 1983a), or in wheatgerm extract (Falkensammer et al., 1985a). The low molecular weight chromogranins are susceptible to immunoprecipitation by antiserum to chromogranin A (Chapter 3), and would therefore presumably be precipitated from the translation products had they been present. Results to the contrary have however, been obtained by Serck-Hanssen and O'Connor (1984). Using antiserum to chromogranin A, they immunoprecipitated 6-10 polypeptides of between

15,000 and 100,000 daltons from translations of bovine adrenal medullary messenger RNA in reticulocyte lysate. The three major polypeptides migrated on SDS-polyacrylamide gels with apparent molecular weights of 100,000, 70,000 and 60,000. These results are most likely due to their antiserum being directed against both chromogranin A and chromogranin B and also to proteolysis during translation. As explained in Section 3.02., it is crucial to separate the chromaffin granule matrix proteins on the basis of their isoelectric points before purifying chromogranin A by electroelution from 1-dimensional SDS-polyacrylamide gels. This ensures that the chromogranin A used for immunizing the rabbits is not contaminated with members of the chromogranin B family. Failure to do this would account for the immunoprecipitation of the 100,000 dalton polypeptide, the precursor to chromogranin B, by O'Connor and Serck-Hanssen. The smaller polypeptides which they immunoprecipitated were probably a result of proteolysis of the 100,000 and 70,000 dalton polypeptides, either during translation or during the subsequent procedure for the immunoprecipitation.

5.03.01.: Further Studies with the Chromogranins

The synthesis of prokaryotic and eukaryotic proteins is initiated with N-formyl methionine and methionine respectively, although only a small percentage of mature proteins have methionine as their NH_2-terminal amino acid. Often N-formyl ^{35}S-methionine-$tRNA_{fmet}$ is used in cell-free translation systems to identify the primary translation product since eukaryotic ribosomes will accept this as the initiator of translation. The presence of

FIGURE 5.03.01.: The fluorograph of a 2-dimensional chromatogram of the DABITC-derived amino-terminal amino acid of the polypeptide immunoprecipitated by antiserum against chromogranin A from a translation of adrenal medullary messenger RNA.



the formyl group inhibits modification of the amino-terminus which might otherwise occur through the action of eukaryotic methionine aminopeptidase and N -acyltransferase, and therefore allows unambiguous identification of the primary translation product.

A much simpler idea was used to establish the fact that the product obtained by immunoprecipitation, after adrenal medullary messenger RNA was translated in reticulocyte lysate, was indeed the primary translation product. This involved performing NH_2-terminal analysis using DABITC (Section 2.05.07.) on the immunoprecipitated polypeptide which had been translated in the presence of ^{35}S-methionine. As explained in Section 5.01.03., the immunoprecipitated putative precursor to chromogranin A can be resolved into two spots by 2-dimensional gel electrophoresis. NH_2-terminal amino acid analyses were not performed individually on these polypeptides. The success of this approach relied on the assumption that the primary translation product would not undergo any NH_2-terminal modifications in the reticulocyte lysate cell-free system, and therefore that the NH_2-terminal amino acid would be identifiable as ^{35}S-methionine.

The immunoprecipitated, radiolabelled polypeptide was electroeluted from a 10% SDS-polyacrylamide gel and subjected to NH_2-terminal amino acid analysis using DABITC (Section 2.05.07.). The resulting fluorograph of the 2-dimensional chromatogram of the DABTH-amino acid revealed the presence of radiolabelled methionine (Fig. 5.03.01.), suggesting that one or both the precursors to chromogranin A were indeed genuine primary translation products.

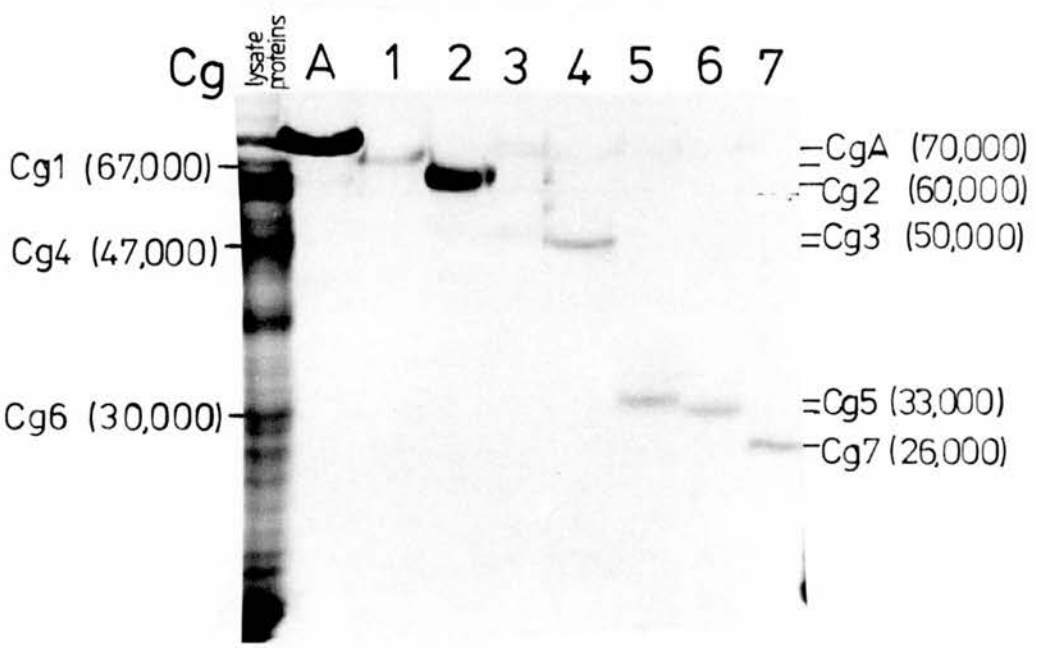


Fig.:- 5.03.02.

1- and 2-dimensional separations of the proteins purified from chromaffin granule matrix proteins. (peak III from DEAE cellulose)

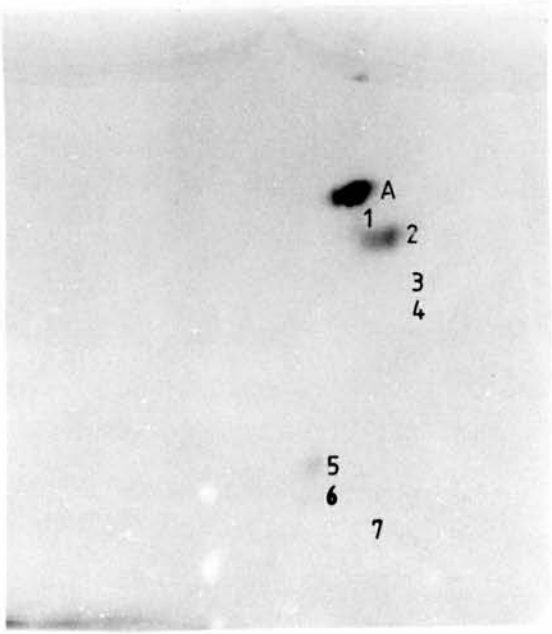
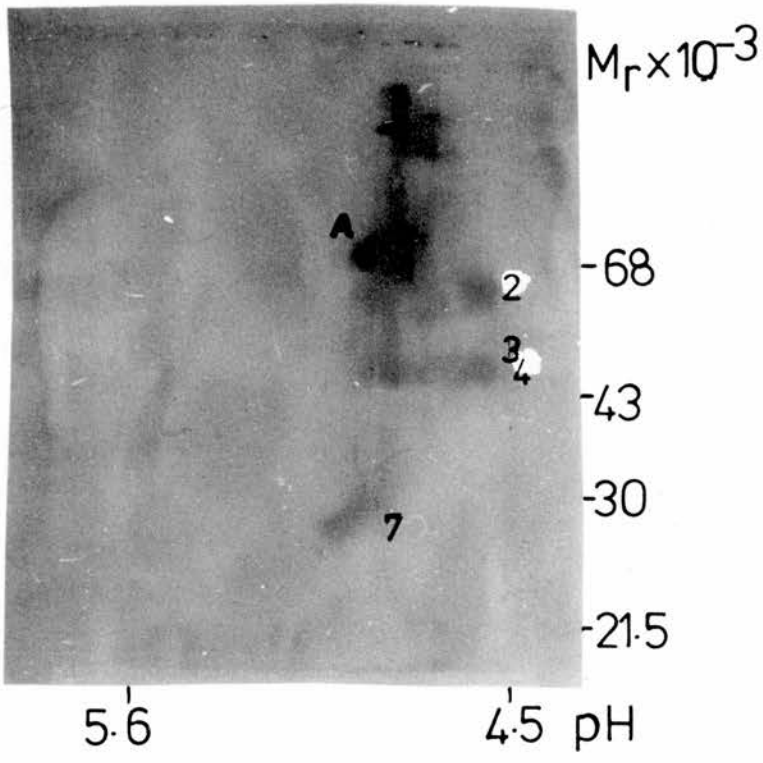


FIG. :- 5.03.03. Immune replica of the purified chromogranins, probed with antiserum against chromogranin A.



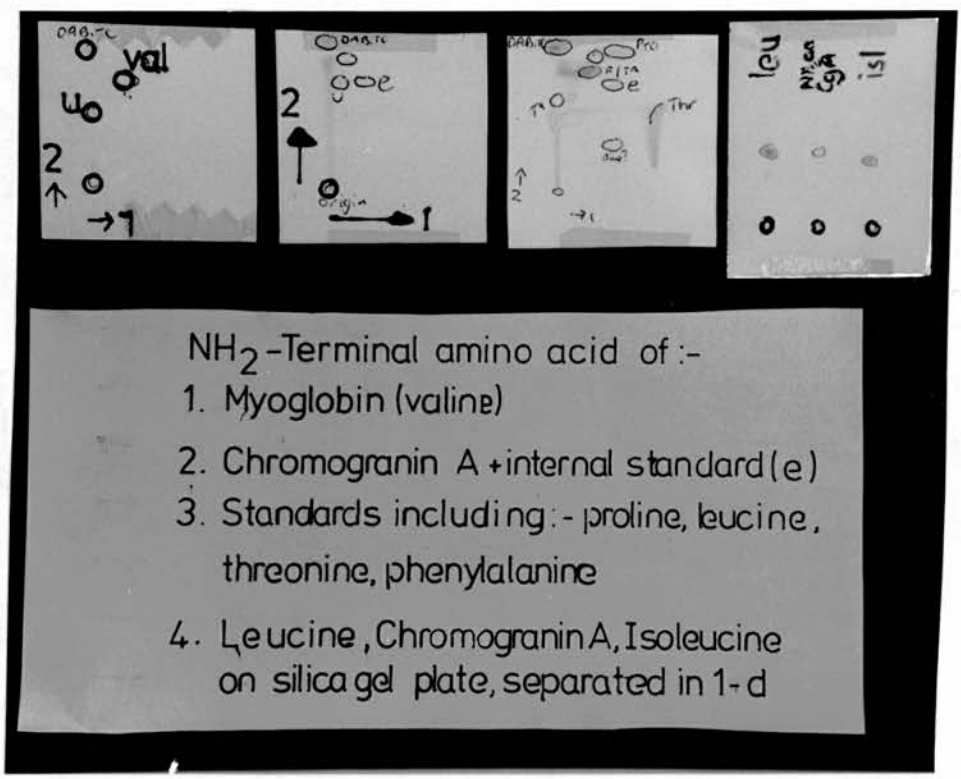
This result was subsequently confirmed by the amino acid sequencing of this immunoprecipitate (Section 5.03.02).

5.03.02.: NH_2-Terminal analysis of Chromogranin A and Several other Chromaffin Granule Matrix Proteins

As discussed in Section 5.02.02., the results obtained from the study of the in vitro and cellular synthesis of chromogranin A suggest that the smaller members of the chromogranin A family arise as a result of proteolysis of chromogranin A. It was therefore of interest to compare the NH_2-terminal sequences of the members of the chromogranin A family with each other and with chromogranin A. Seven acidic proteins from the chromaffin granule matrix were purified by running the polypeptides from peak 3, obtained from DEAE cellulose chromatography of chromaffin granule lysate (Section 2.02.03.), on 10% SDS-polyacrylamide gels followed by the excision of the Coomassie-stained bands and electroelution (Section 2.04.06.). After dialysis to remove residual SDS, the purified proteins were examined by 1- and 2- dimensional gel electrophoresis (Fig. 5.03.02.) and tested for their reactivity with antiserum to chromogranin A by immune-blotting (Section 2.04.05.; Fig.5.03.03). Two of the purified proteins, chromogranins 5 and 6, were not recognized by this antiserum, and were therefore not further studied. The other proteins were immunologically related to chromogranin A and they, along with chromogranin A were subjected to NH_2-terminal amino acid analysis using DABITC (Section 2.05.07.). The NH_2-terminal amino acid of chromogranin A was determined as leucine (Fig.5.03.04), as was that of chromogranins 2 and 4. The attempts to determine the

FIGURE 5.03.04.:

2-dimensional chromatograms of standard DABTH-derivatives and the amino-terminal amino acid of myoglobin on polyamide chromatography sheets, and a 1-dimensional chromatogram on a silica gel plate of the amino-terminal amino acid of chromogranin A



NH_2-terminal amino acid of chromogranins 1 and 3 were unsuccessful. Attempts to determine the second amino acid from the NH_2 terminus of chromogranin A and chromogranins 2 and 4 were also unsuccessful.

The result obtained however, was potentially interesting in that it suggested the possibility that chromogranin A and several of the related chromogranins had identical NH_2-termini. This in turn suggested that the protease(s) acting on chromogranin A did so nearer the carboxyl than the amino terminus. However, it was obvious that NH_2-terminal sequence analysis using DABITC was not, in this instance, a satisfactory method for the determination of the sequence of the first few residues of these proteins. Samples of chromogranin A and chromogranins 1 and 3, were therefore sent to the Department of Biochemistry, University of Aberdeen, Scotland, for sequence analysis by gas phase Edman degradation coupled with HPLC analysis of the PTH-amino acids. The first 24 NH_2-terminal amino acids of chromogranin A had previously been determined (Hogue-Angeletti, 1977) and compared with the amino acid sequence of the first 20 residues of parathyroid secretory protein I (Cohn et al., 1982). These were found to differ at only two residues in positions 2 and 19. Recently thefirst 19 NH_2-terminal amino acids of chromogranin A were resequenced and found to be identical to parathyroid secretory protein I (Kruggel et al., 1985). For some unknown reason, large losses of material occurred during each cycle of degradation of my chromogranin A and therefore only the first ten NH_2-terminal residues were determined. The results are compared with the other published sequence data for chromogranin A in Fig. 5.03.05.. Unfortunately, no sequence data could be obtained for the

Fig.: 5.03.05.: COMPARISON OF AMINO ACID SEQUENCES OBTAINED FOR CHROMOGRANIN A AND PARATHYROID SECRETORY PROTEIN I

-1- -2- -3- -4- -5- -6- -7- -8- -9- 10- 11- 12- 13- 14 -15 -16 -17 -18 -19 -20-

Chromogranin A H_2N-Leu-Arg-Val-Asn-Ser-Pro-Met-Asn-Lys-Gly-Asp-Thr-Glu-Val-Met-Lys-Cys-Ile-Arg-Glu-

(from Hogue-Angeletti, 1977)

Secretory Protein I H_2N-Leu-Pro-Val-Asn-Ser-Pro-Met-Asn-Lys-Gly-Asp-Thr-Glu-Val-Met-Lys-XXX-Ile-Val-Glu-

(from Cohn et al., 1981)

Chromogranin A H_2N-Leu-Pro-Val-Asn-Ser-Pro-Met-Asn-Lys-Gly-Asp-Thr-Glu-Val-Met-Lys-Cys-Ile-Val-Glu-

(from Kruggel et al., 1985)

Chromogranin A H_2N-Leu-Pro-Val-Asn-Ser-Pro-Met-Asn-Lys-Gly-

(sequence obtained for chromogranin A at the University of Aberdeen)

chromogranins 1 and 3. Sequence analysis of the ^{35}S-methionine labelled polypeptide which was immunoprecipitated by antiserum to chromogranin A from the translation products (Section 5.01.02) was also performed at Aberdeen University. The results are shown in Fig. 5.03.05. and clearly confirm the result obtained by DABITC (Section 5.03.01.) that one or both of the NH_2-terminal polypeptides are radiolabelled and that therefore the immunoprecipitate is likely to be the genuine primary translation product. The results also suggest that residues 9 and 16 of this polypeptide are radiolabelled.

During the course of this work, the chromogranin B family was identified and the problems involved in the purification of chromaffin granule matrix proteins to homogeneity were realized. Ideally the proteins, particularly the lower molecular weight proteins, would have had to have been electroeluted from 2-dimensional gel separations of the peak 3 obtained from DEAE-cellulose chromatography of the matrix proteins (Fig.:3.02.). Due to the problems which would be involved in the purification of sufficient quantities of these matrix proteins to homogeneity, and to the potential problems of gas phase sequencing of these proteins, this project was not pursued any further. Gene sequence analysis of chromogranin A, which is already underway in the U.S.A., should identify potential sites for proteolytic cleavage on the protein and may give insight into the polypeptides generated from chromogranin A. Sequence analysis may also reveal the presence of already identified biologically active neuropeptides within the sequence of chromogranin A, and similar possibilities exist for the chromogranin B family.

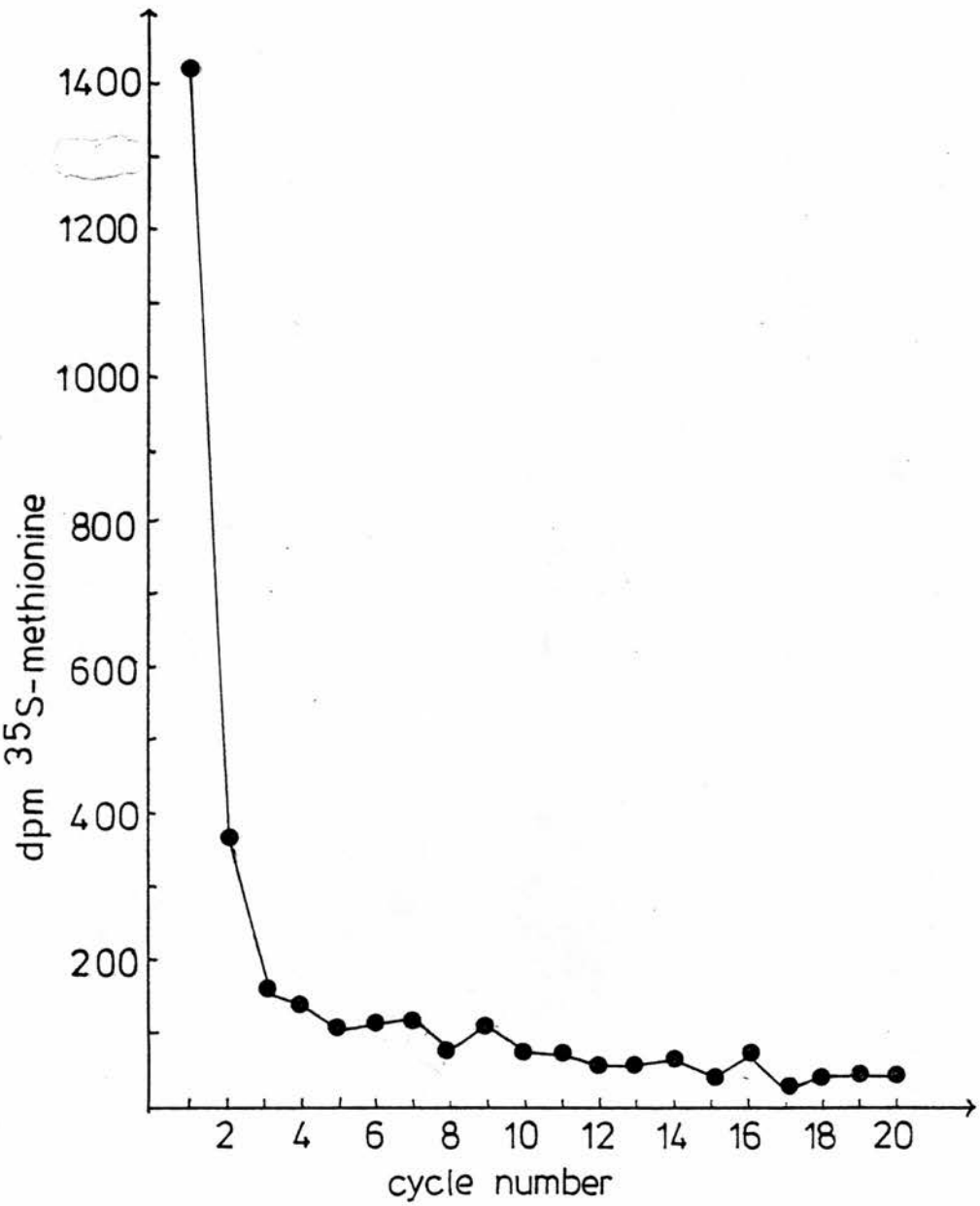


Fig.: 5.03.06. DPM of radioactivity per cycle of Edman degradation of the polypeptide immune-precipitated by antiserum to chromogranin A from translation products

However, although preliminary, the results obtained in this study suggest the possibility that proteolysis of chromogranin A may occur from nearer the carboxyl terminus of the protein to produce smaller proteins with the same amino terminus. This has also been suggested by the preliminary sequencing data of Settleman et al., 1985), who have sequenced the amino terminal amino acids of chromogranin A and several other smaller chromogranins.

Defined degradation from the carboxyl terminus has been identified as one of the ageing processes of the vertebrate eye protein, α-crystallin (Bloemandel, 1977). Similarly, proteolysis of chromogranin A which, as discussed previously (Section 5.02.02.) appears to be a relatively slow process, could be a result of ageing. The function of chromogranin A is unknown as is the significance of the proteolytic processing. The importance of proteolysis in neuropeptide processing has only recently been realized, but it is clear that proteolytic post-translational modification is of common occurrence in the generation of biologically active peptides and proteins from larger precursors. Two of the best-characterized neuropeptide proproteins are pro-opiocortin and pro-enkephalin (Loh et al., 1984). As discussed in Section 1.08.02., it is possible that the trypsin-like protease (Lindberg et al., 1984) and the cobalt-stimulated carboxypeptidase (Fricker and Snyder, 1982), which are present within the chromaffin granule matrix and are probably responsible for the processing of proenkephalin, may also be involved with the processing of chromogranin A. However, there may be other, as yet unidentified protease activities present within the chromaffin

granule responsible for the proteolytic processing of matrix proteins such as chromogranins A and B.

CHAPTER 6

STUDIES WITH DOPAMINE β-HYDROXYLASE AND CYTOCHROME B_{561}

RESULTS AND DISCUSSION

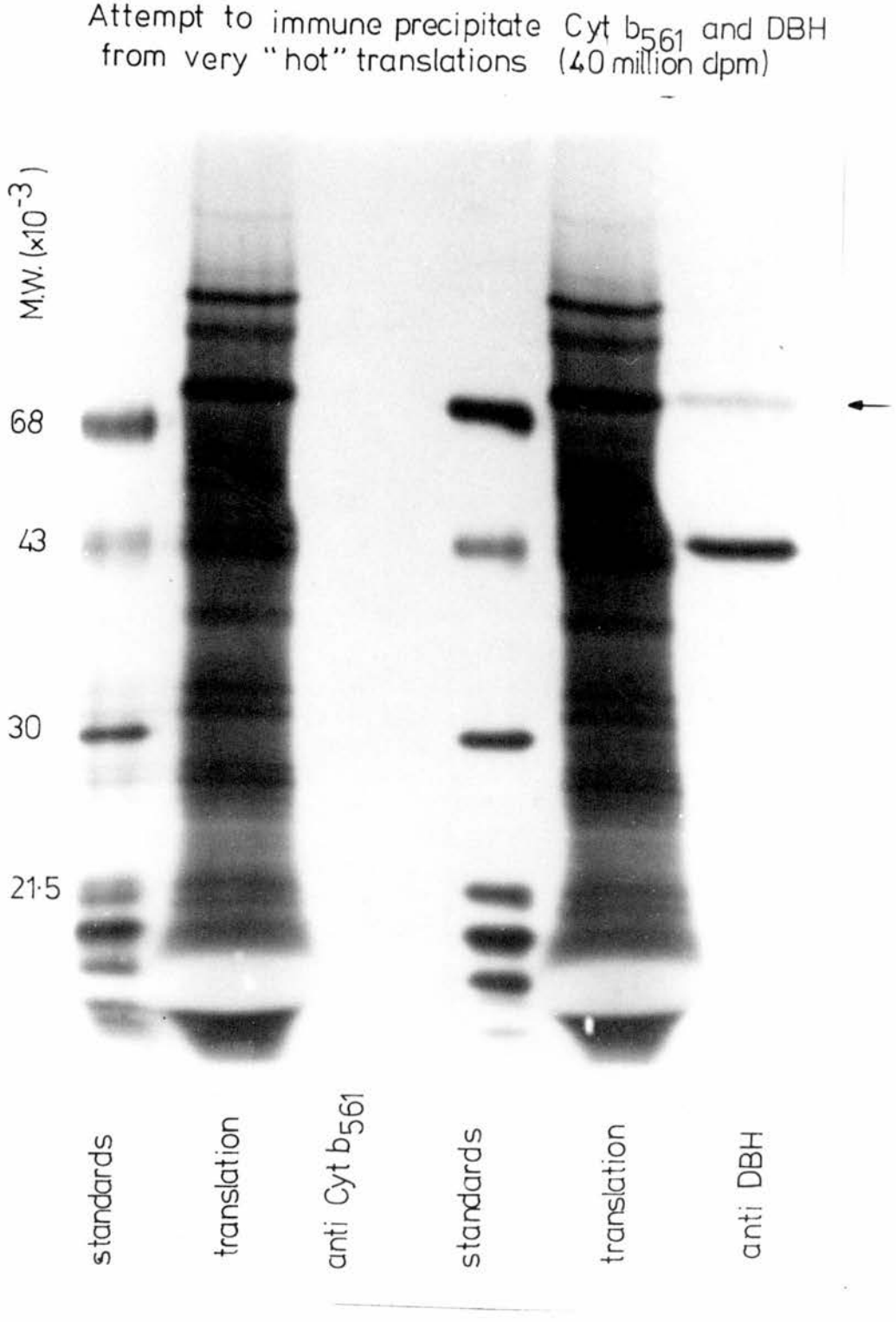


Fig.:-6.01.01.

(See 2.03.07. for details of translations). Immunoprecipitations were performed as described in 2.03.12. and fluorographs were exposed for 4 days at -70°C.

6.01.:Further Attempts to Characterize the Primary Precursors to Dopamine β-Hydroxylase and Cytochrome b_{561}

As mentioned previously (Section 4.09.), newly-synthesized polypeptides from a translation mix must incorporate sufficient radiolabelled amino acid in order to be detectable by fluorography. From translations performed in a final volume of 52µl, containing about $6x10^6$ dpm of ^{35}S-labelled TCA-precipitable material, it was possible to detect the translation product immunoprecipitated by antiserum to chromogranin A, whereas the antisera to dopamine β-hydroxylase and cytochrome b_{561} did not appear to immunoprecipitate any translation products (Section 5.01.). However, it was possible that translation products immunoprecipitated by these antisera had not incorporated enough radiolabel to be detected. For this reason, an attempt was made to immunoprecipitate dopamine β-hydroxylase and cytochrome b_{561} from translations of 312µl, containing about $40x10^6$ dpm of TCA-precipitable material. This increased the chance considerably of being able to detect the immunoprecipitated polypeptides by fluorography, simply because there would be more radiolabelled polypeptide present.

6.01.02.: Immune-Precipitation of Translation Products Recognized by Dopamine β-Hydroxylase and Cytochrome b_{561}

When adrenal medullary messenger RNA was translated in message-dependent reticulocyte lysate reactions of 312µl (Section 2.03.04.), followed by immunoprecipitation (Section 2.03.12.) using

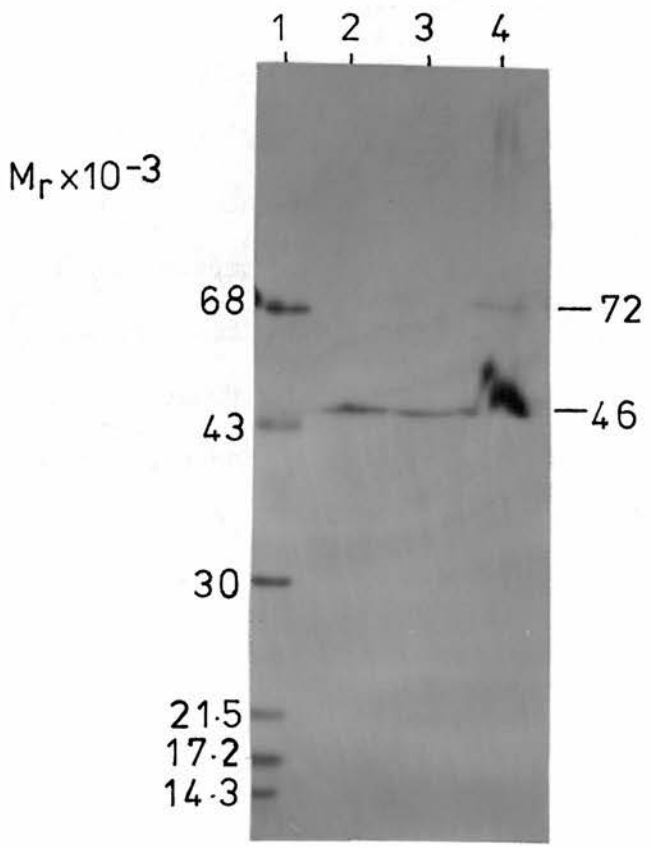
FIGURE 6.01.02.: Competition Experiment

Purified dopamine β-hydroxylase (20µg) was added to translation products to compete with translation products for immunoglobulins against dopamine β-hydroxylase.

Lane 1 : Molecular Weight Standards

Lane 2,3 : Immunoprecipitation of dopamine β-hydroxylase from translation products, performed in the presence of purified dopamine β-hydroxylase

Lane 4 : Immunoprecipitation of dopamine β-hydroxylase from translation products



antiserum to soluble dopamine β-hydroxylase or cytochrome b_{561}, the resulting fluorograph of a SDS-polyacrylamide gel (Fig.6.01.01.) revealed that the antiserum to dopamine β-hydroxylase recognized translation products migrating with apparent molecular weights of 72,000 and 46,000. The antiserum to cytochrome b_{561} did not, however, precipitate any detectable translation product. About 0.3% of the total TCA-precipitable material from the translation reaction was precipitated by the antiserum to soluble dopamine β-hydroxylase. Of the precipitated material there was between 2-4 times more radiolabel present in the 46,000 dalton polypeptide than there was in the 72,000 dalton polypeptide, however this varied between experiments.

In order to determine whether both the 72,000 and 46,000 dalton polypeptides were genuinely recognized by anti dopamine β-hydroxylase immunoglobulins, excess (20μg) soluble dopamine β-hydroxylase, prepared by chromatography on DEAE cellulose and concanavalin A sepharose (Sections 2.02.03. and 3.02), was added to an immunoprecipitation reaction to compete against the translation products for the antibody binding sites. As shown in Fig. 6.01.02., the addition of excess dopamine β-hydroxylase completely inhibited the precipitation of the 72,000 dalton polypeptide and substantially reduced the precipitation of the 46,000 dalton polypeptide, thereby suggesting that both these polypeptides are immunologically related to dopamine β-hydroxylase.

6.01.03.: Further Studies with the Translation Products Recognized by Antiserum to soluble Dopamine β-Hydroxylase

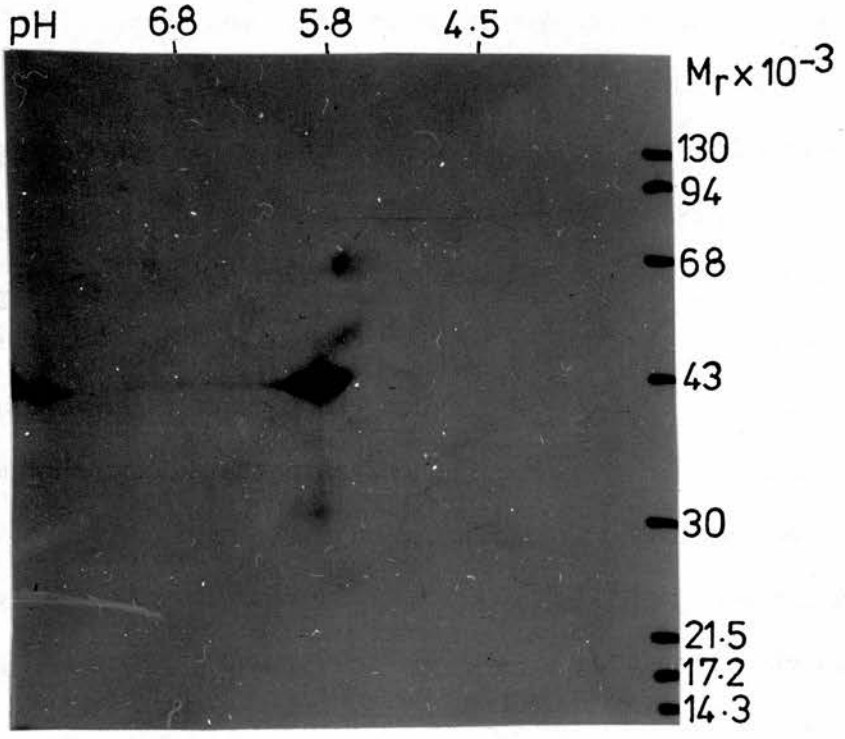
As discussed in Section 1.10.02., dopamine β-hydroxylase is a tetramer comprising four similar subunits of 75,000 daltons. It is reasonable to assume that the 72,000 dalton polypeptide translation product recognized by the antiserum to dopamine β-hydroxylase, could be a precursor to a 75,000 dalton subunit. However, it is difficult to understand why this antiserum also recognizes a 46,000 dalton translation product. There are three possible explanations as follows:

1. The dopamine β-hydroxylase, used to immunize the rabbits, was contaminated and the resulting antiserum therefore contains antibodies to the 46,000 dalton contaminant in addition to dopamine β-hydroxylase. However two different rabbit antisera, one against dopamine β-hydroxylase prepared by chromatography on DEAE cellulose and concanavalin A sepharose (Sections 2.02.03. and 3.02.), and the other prepared against dopamine β-hydroxylase purified by chromatography followed by electroelution from a one dimensional SDS-polyacrylamide gel, run under non-reducing conditions, have been used for the immunoprecipitations. Both antisera immunoprecipitate the 72,000 and 46,000 dalton polypeptides from translations suggesting that, if a contaminant is present in the immunizing preparation of dopamine β-hydroxylase, it will prove difficult to remove.

2. Sabban and Goldstein (1984) immunoprecipitated polypeptides of 67,000 and 32,000 daltons from translations performed in wheatgerm

FIGURE 6.01.03.:

Fluorograph of a 2-dimensional gel of the polypeptides immunoprecipitated from translation products by antiserum to dopamine β-hydroxylase



extract, of adrenal medullary bound polysomal messenger RNA using antiserum to dopamine β-hydroxylase. The 67,000 dalton and 32,000 dalton polypeptides were, they believe, the precursors to dopamine β-hydroxylase and phenylethanolamine N-methyl transferase respectively. Their bound polysome preparation was contaminated with free polysomes and hence phenylethanolamine N-methyl transferase was synthesized. These results support the proposal that the enzymes involved with the biosynthesis of catecholamines contain homologous polypeptide domains (Ross et al., 1982). This has been further supported with evidence suggesting the presence of homologous coding regions in the genes for these enzymes (Joh et al., 1983). However, the polypeptides immunoprecipitated with antiserum to dopamine β-hydroxylase using reticulocyte lysate to translate total adrenal medullary messenger RNA, are clearly different from those immunoprecipitated by Sabban and Goldstein. The 46,000 dalton polypeptide is unlikely to be equivalent to the 32,000 dalton polypeptide, the precursor to phenylethanolamine N-methyl transferase, although it is conceivable that the discrepancy is a result of the different translation systems used.

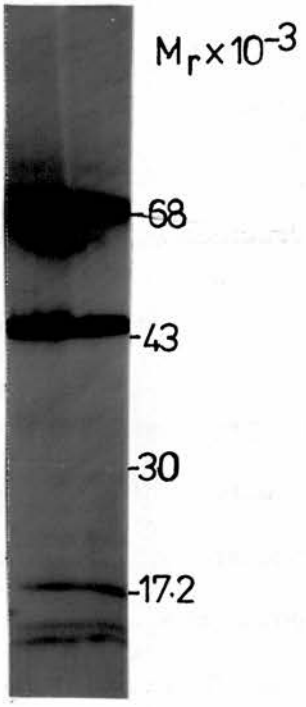
3. A third possibility is that the 46,000 dalton polypeptide is a proteolytic breakdown product of the 72,000 dalton polypeptide and this may be supported by the following evidence. When separated by 2-dimensional gel electrophoresis (Fig.6.01.03.) the 72,000 and 46,000 dalton polypeptides have the same pI suggestive of a relationship between the two, as is the case with the proteolytic products of chromogranin A. Also, when dopamine β-hydroxylase is precipitated from a translation performed in the presence of membranes (Section 6.02.01), as much, if not more translation product

is precipitated (Fig. 6.02.01.), despite there being about 30% less radiolabelled TCA-precipitable material since translation is less efficient in the presence of microsomes (Section 4.06.02.). In this case, of the precipitated material, approximately 4 times more radiolabel was present in the 67,000 dalton polypeptide than there was in the 46,000 dalton polypeptide. This could be due the newly-synthesized and translocated dopamine β-hydroxylase being protected by the microsomal membranes from protease activity, which may be present. Alternatively however, the polypeptide may become more stable after signal sequence cleavage and/or disulphide bond formation. Glycosylation of polypeptides also confers a degree of protease resistance on the polypeptide chain: the unglycosylated form can be extremely sensitive to proteases (Olden et al., 1982) and it is possible that the primary precursor to dopamine β-hydroxylase falls into this category.

If the 46,000 dalton polypeptide is a proteolytic fragment of

FIGURE 6.01.05.:

Overexposed fluorograph revealing polypeptides of low molecular weight which are immunoprecipitated from translation products by antiserum to dopamine β-hydroxylase.



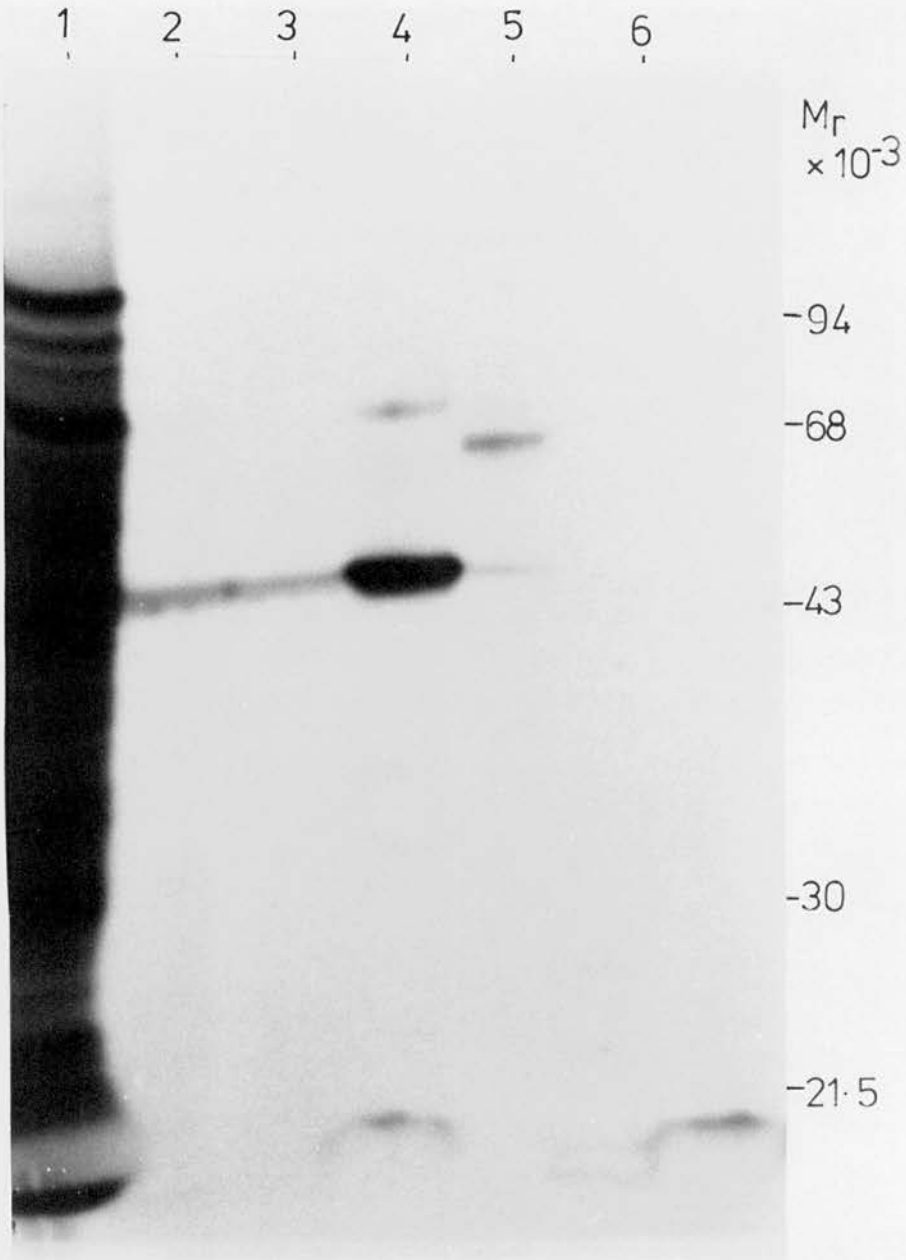
Fluorograph was exposed for 21 days @ -70°C.

the 72,000 dalton polypeptide, where is the remaining 26,000 dalton fragment? Over-exposure of a fluorograph of the translation products immunoprecipitated by antiserum to dopamine β-hydroxylase reveals a small amounts of a 33,000 dalton polypeptide but larger amounts of three low molecular weight polypeptides migrating with apparent molecular weights of 17,000, 12,000 and 10,000 daltons (Fig. 6.01.05). These could account for the "missing" 26,000 dalton polypeptide. Although the evidence presented in favour of the 46,000 dalton polypeptide being a proteolytic product of the 72,000 dalton polypeptide is weakened if the polypeptides are different proteins with some homologous domains, it is impossible to reconcile the results obtained here with those of Sabban and Goldstein (1984).

6.01.03.: Cotranslational Processing of Dopamine β-Hydroxylase

Translation reactions (312μl) of adrenal medullary messenger RNA were performed in the presence of dog pancreas microsomes. Approximately 28×10^6 dpm were incorporated into TCA-precipitable material. Microsomal vesicles were isolated by centrifugation (Section 2.03.08.) and antiserum to soluble dopamine β-hydroxylase was used to immunoprecipitate (Section 2.03.12.) microsome associated translation products. The fluorograph of a one-dimensional SDS-polyacrylamide gel (Fig. 6.01.06.) revealed the presence of a polypeptide migrating with an apparent molecular weight of 67,000 daltons. The 72,000 and 46,000 dalton polypeptides were just visible and may have been due to contamination with untranslocated translation products. After translation in the presence of microsomes to which chymotrypsin and trypsin had been added, the

Fig.:-
6.01.06.



1. Translation 2, 3. S. aureus cells added to translation
4. DBH from translation
5. " " " in the presence of microsomes.
6. preimmune serum added to translation.

Immunoprecipitations were performed as described in 2.03.12. Fluorograp
were exposed for **4 days @ -70°C.**

67,000 dalton polypeptide product was still immunoprecipitable so must therefore have been translocated across the microsomal membrane (Section 4.06.02.).

The results so far described are consistent with the dopamine β-hydroxylase subunit being synthesized as a precursor of 72,000 daltons which, when synthesized in the presence of microsomal membranes loses a signal sequence of around 5,000 daltons thus generating a polypeptide of 67,000 daltons. However, as discussed in Section 1.10.02., the subunits of dopamine β-hydroxylase have N-glycosidically linked carbohydrate (Fischer-Colbrie et al., 1982; Gavine et al., 1984; Margolis et al., 1984). Some preparations of dog pancreas microsomes are capable of cotranslational core glycosylation (Walter and Blobel, 1983b). It was therefore important to determine whether the 67,000 dalton polypeptide was core-glycosylated or not since this would alter the molecular weight considerably.

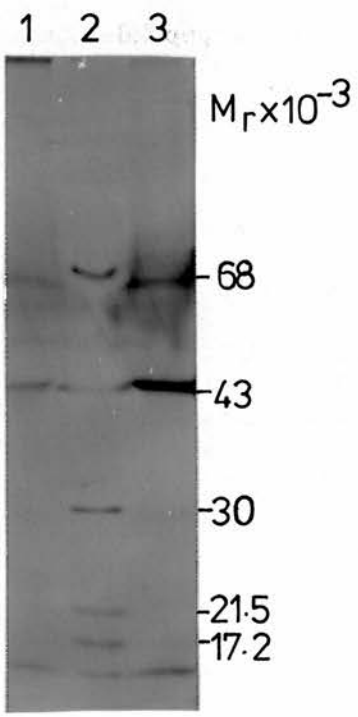
Nuclease-treated microsomes were made 0.1% in $C_{12}E_8$ immediately before their addition to a translation reaction. The detergent disrupts the lipid bilayer, thus preventing cotranslational core glycosylation, but does not affect signal sequence cleavage (Jackson and Blobel, 1980). The polypeptide immunoprecipitated from the translation performed in the presence of the $C_{12}E_8$ treated microsomes co-migrated with the 67,000 dalton polypeptide immunoprecipitated from a translation performed in the presence of untreated microsomes (Fig.6.01.07). This suggests that the 67,000 dalton polypeptide is the proteolytically processed but unglycosylated precursor to the mature subunit of dopamine β-hydroxylase. The putative signal

FIGURE 6.01.07.: Fluorograph of

Lane 1. : The polypeptide immunoprecipitated by antiserum to dopamine β-hydroxylase from the products of a translation, performed in the presence of dog pancreas microsomal membranes

Lane 2. : M_r Standards

Lane 3. : The polypeptide immunoprecipitated by antiserum to dopamine β-hydroxylase from the products of a translation, performed in the presence of $C_{12}E_8$-treated dog pancreas microsomal membranes



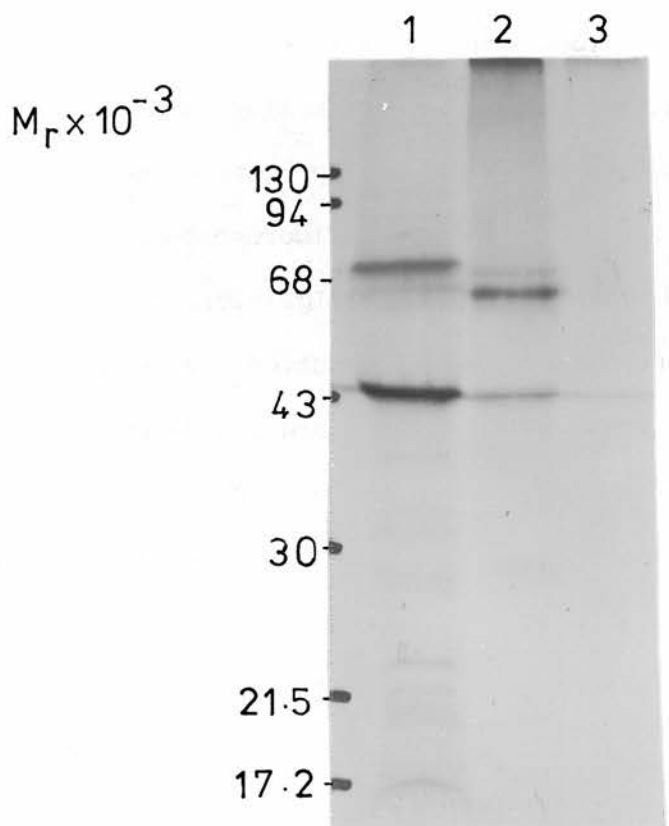
sequence of the primary translation product is therefore rather large at 5,000 daltons i.e. about 40 amino acids. The putative unglycosylated precursor to dopamine β-hydroxylase, immunoprecipitated from the polypeptide products of a translation performed in the presence of microsomal membranes, is somewhat smaller than chemically deglycosylated dopamine β-hydroxylase which migrates as a doublet on SDS-polyacrylamide gels with apparent molecular weights of 72,000 (Apps et al., 1985). Chemically deglycosylated dopamine β-hydroxylase does however, contain residual asparagine-linked N-acetyl glucosamine since it still binds the lectin wheatgerm agglutinin. One other discrepancy between the results obtained from in vitro translation experiments and the chemical deglycosylation of dopamine β-hydroxylase is the apparent pI of the polypeptide. The polypeptides immunoprecipitated by antiserum to dopamine β-hydroxylase from translations in the presence or absence of microsomal membranes are more acidic than "mature" dopamine β-hydroxylase. However, chemically deglycosylated dopamine β-hydroxylase is, as would be expected, more basic than "mature" dopamine β-hydroxylase (D.K. Apps, unpublished results). The post-translational processing event(s) which would result in the precursor becoming more basic can only be guessed at. However, preliminary results from the cellular synthesis of dopamine β-hydroxylase suggest that the shift towards a higher pI is indeed a genuine post-translational modification (J.G. Pryde, unpublished results). Glycosylated dopamine β-hydroxylase, especially the membrane-bound form, does not focus as a sharp band after isoelectric focusing, nor does it fully enter the SDS-polyacrylamide gel. The discrepancy of apparent pI values obtained may therefore be an

FIGURE 6.01.08.: Immunoprecipitation of Dopamine β-Hydroxylase from:-

Lane 1 : a translation

Lane 2 : the aqueous phase from a Triton X-114 separation of microsomal vesicles, isolated from a translation performed in the presence of dog pancreas microsomal membranes

Lane 3 : the detergent-rich phase from a Triton X-114 separation of microsomal vesicles, isolated from a translation performed in the presence of dog pancreas microsomes



artefact of 2-dimensional gel electrophoresis.

6.01.04.: The 67,000 dalton Polypeptide is Translocated into the Lumen of Microsomal Vesicles

As discussed in Section 1.10.02. evidence is accumulating that the membrane-bound and soluble forms of dopamine β-hydroxylase have different subunit compositions (Skotland et al., 1977; Saxena and Fleming, 1983; Sokoloff et al., 1985). However, the nature of the difference is unknown. In order to determine whether the immunoprecipitated 67,000 dalton translation product from a translation in the presence of membranes remained anchored in the membrane or was translocated into the lumen of the microsomal vesicles, the vesicles from a translation reaction were isolated by centrifugation (Section 2.03.08.) and subjected to phase separation in Triton X-114 (Section 2.03.14.). Antiserum to dopamine β-hydroxylase was added to the resulting membrane and aqueous fractions and the normal procedure for immunoprecipitation carried out (Section 2.03.12.) The resulting fluorograph of a 1-dimensional SDS-polyacrylamide gel is shown in Fig. 6.01.08. The 67,000 dalton polypeptide is found in the aqueous fraction. When the proteins of whole chromaffin granule membranes are separated in Triton X-114, dopamine β-hydroxylase is found in two fractions - the aqueous fraction and the detergent-rich fraction (Pryde and Phillips, 1985), representing the soluble and membrane-bound forms respectively. It is therefore probable that only the soluble form of dopamine β-hydroxylase is being synthesized in large enough amounts to be detected in these studies.

6.01.05.: The Biosynthetic Relationship between the Membrane-Bound and Soluble Forms of Dopamine β-Hydroxylase

Limited proteolysis experiments have suggested that membrane-bound dopamine β-hydroxylase is the precursor to soluble dopamine β-hydroxylase (Bjerrum et al., 1979; Helle et al., 1984). This suggestion has been further substantiated by radiolabelling and immunoprecipitation of dopamine β-hydroxylase from rat pheochromocytoma cells (Sabban et al., 1983). These experiments suggest that the primary translation product is a 67,000 dalton polypeptide which is rapidly glycosylated in vivo to a 77,000 dalton membrane-bound form of the enzyme. Pulse-chase experiments suggest that the 77,000 dalton polypeptide is then processed to the 73,000 dalton soluble form. However, different results have been obtained by McHugh et al., (1985) who, using the same system, have found that the 77,000 and 73,000 dalton polypeptides are synthesized simultaneously. Both types of subunit occur in the membrane-bound enzyme whereas the soluble form only contains the 73,000 dalton polypeptide. These results suggest that the two forms are synthesized individually as proposed by Winkler (1977) and as suggested by pulse-labelling experiments with perfused adrenal glands (Ledbetter et al., 1978). Interpretation of these results has however been further complicated by the identification of a third form of dopamine β-hydroxylase from rat pheochromocytoma which is sulphated and secreted by a constitutive pathway (M^{C}Hugh et al., 1985). Results obtained from rat pheochromocytoma cells are not, however, automatically applicable to the situation in intact adrenal glands.

6.01.06.: Biosynthesis of Soluble Dopamine β-Hydroxylase *in vitro*

In this study, the biosynthesis of the soluble form of dopamine β-hydroxylase is most likely being observed since the 67,000 dalton polypeptide was immunoprecipitated from the aqueous phase after Triton X-114 phase separation of the microsomal vesicles from a translation (Section 6.01.04). This tends to support the proposal that the two forms of the enzyme are synthesized individually (Winkler, 1977; Ledbetter *et al.*, 1978; M^{c}Hugh *et al.*, 1985) and is at least inconsistent with the proposal that the membrane-bound form is the precursor of the soluble form (Sabban *et al.*, 1983; Helle *et al.*, 1984). These results suggest that the 72,000 dalton primary precursor of the soluble enzyme is processed during translocation to a 67,000 dalton polypeptide. It is possible that N-linked glycosylation of this 67,000 dalton polypeptide increases its molecular weight to the 73,000 as found for the soluble enzyme in rat pheochromocytoma cells (M^{c}Hugh *et al.*, 1985). The membrane bound form of dopamine β-hydroxylase may well not be present in large enough quantities in the translation reaction to be detected. As will be discussed in Chapter 8, this may be due to membrane recycling which would reduce the need for the cell to synthesize membrane bound dopamine β-hydroxylase. On the other hand, the soluble form of the enzyme is constantly having to be synthesized to replenish the dopamine β-hydroxylase lost by exocytosis.

6.01.07.: An Alternative Explanation for the Results obtained for Dopamine B-hydroxylase

Signal sequences responsible for directing polypeptides to the endoplasmic reticulum are normally 15-30 amino acids long. The apparent size of the signal sequence on the putative primary precursor to dopamine β-hydroxylase is rather large, about 40 amino acids. It is possible to interprete the results presented above in a different way.

Semliki forest virus glycoproteins E_1 and E_2 (Uterman and Simons, 1974) and cytochrome b_5 (Spatz and Strittmatter, 1971) are anchored into membranes by hydrophobic amino acid sequences of 40 amino acids. These proteins are synthesized with an NH_2-terminal signal sequence which is cotranslationally cleaved. They are anchored into the membrane by halt-transfer sequences at their carboxyl terminals.

Is it possible that membrane-bound dopamine β-hydroxylase is anchored into the chromaffin granule membrane by a sequence of 40 amino acids which, under these <u>in vitro</u> circumstances is cleaved, thus releasing a smaller (67,000 dalton) polypeptide into the lumen of the dog pancreas microsomes?

Clearly many of the solutions to the problems regarding the biosynthesis of dopamine β-hydroxylase await cloning and DNA sequencing of the gene(s) encoding this enzyme.

6.02.01.:Further Studies with Cytochrome b_{561}

As mentioned above (Section 6.01.02), initial attempts to immunoprecipitate newly-synthesized peptides, using an antiserum directed against cytochrome b_{561} were unsuccessful. The antiserum originally prepared against detergent-solubilized cytochrome b_{561} recognized only a small cytoplasmic domain of the cytochrome b_{561} (Hunter et al., 1982) even though it is a transmembrane protein. It was therefore possible that the failure to immunoprecipitate the precursor to cytochrome b_{561} from in vitro translation reactions was due to the antiserum being directed against only a small domain of the protein. This prompted the preparation of various modified forms of cytochrome b_{561} against which rabbit antisera were produced.

6.02.02.: Preparation of Antisera to Chymotrypsin-cleaved and Papain-cleaved Cytochrome b_{561}

Incubation of freshly prepared, intact chromaffin granules (Section 2.02.06.) with proteases, results in the cleavage of the cytoplasmically exposed domains of granule membrane proteins, including cytochrome b_{561} (Abbs and Phillips, 1980; Hunter et al., 1982). Chromaffin granules were incubated with either chymotrypsin or papain (Section 2.02.06.) and the membrane protected domain of cytochrome b_{561} was purified as described in Section 2.02.05.. Cytochrome b_{561}, which migrated on 12.5% SDS-polyacrylamide gels with an apparent molecular weight of 27,500 was reduced to polypeptides of 25,000 and 23,500 respectively when granules were

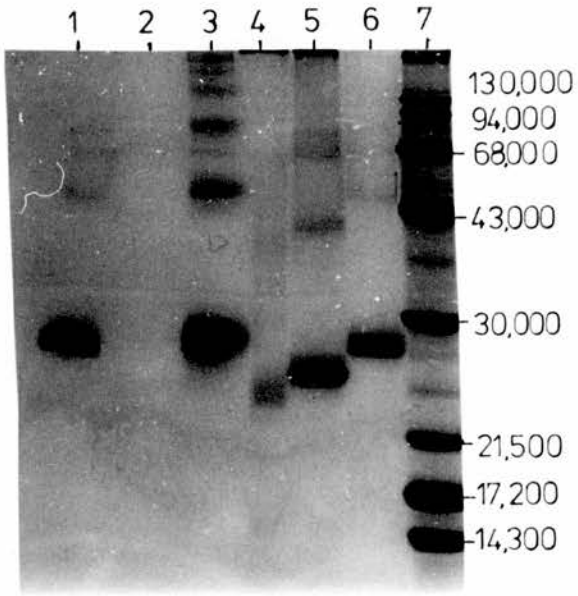


Fig.: 6.02.01. LANE 1 Cytochrome b_{561}
2 CnBr fragments of cyt. b_{561}
3 Carboxymethylated cyt. b_{561}
4 Papain-cleaved cyt. b_{561}
5 Chymotrypsin-cleaved cyt. b_{561}
6 Cytochrome b_{561}
7 M_r Standards

12·5% SDS-Polyacrylamide gel

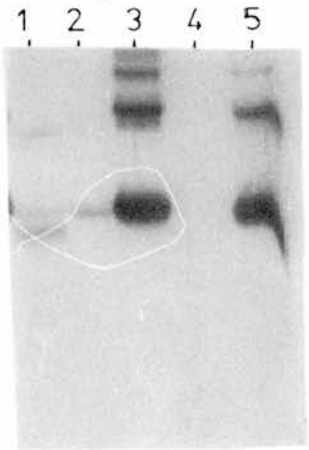


Fig.: 6.02.02. Immune-replica using antiserum to carboxymethylated cytochrome b_{561} against
LANE 1 chymotrypsin-cleaved cyt. b_{561}
2 papain-cleaved cyt. b_{561}
3 carboxymethylated cyt. b_{561}
4 CnBr fragments of cyt. b_{561}
5 cytochrome b_{561}

treated with chymotrypsin and papain (Fig. 6.02.01.). However, immune replicas revealed that trace amounts of non-cleaved cytochrome b_{561}, which was recognized by the original antiserum to cytochrome b_{561}, were present in the protease-treated cytochrome preparations. This antiserum did not, however, recognize the protease-cleaved forms of cytochrome b_{561}. Chymotrypsin-cleaved and papain-cleaved cytochromes b_{561} were therefore electroeluted (Section 2.04.06.) from 12.5% SDS-polyacrylamide gels to remove all traces of non-cleaved cytochrome b_{561} from the preparation. The purified chymotrypsin-cleaved and papain-cleaved cytochrome b_{561} preparations were used to immunize New Zealand white rabbits as described in Section 2.05.01., in an attempt to produce antisera which recognized different antigenic sites on the protein.

6.02.03.: Preparation of Antiserum to Reduced and S-carboxymethylated cytochrome b_{561}

Often antisera produced against non-reduced proteins do not recognize the polypeptide precursors from *in vitro* translations (Counis *et al.*, 1981). Cytochrome b_{561} has four cysteine residues and therefore possibly two disulphide bonds (Duong and Fleming, 1982). It was possible that irreversible cleavage of the disulphide bonds by reduction and S-carboxymethylation would unfold the protein and reveal different antigenic determinants. Reduced and S-carboxymethylated cytochrome b_{561} was therefore prepared as described in Section 2.02.07. and was used to immunize a New Zealand white rabbit (Section 2.05.01.).

Table 6.02.

Summary of the effectiveness of the antisera prepared against various modified forms of Cytochrome b_{561}

Anti-serum against	PROTEIN				
	cyt	CM-Cyt	CnBr-Cyt	CT-cyt	Pap-Ct
cyt	strong	strong	-ve	-ve	-ve
CM-cyt	very srong	very strong	-ve	strong	weak
CnBr-cyt	weak	weak	-ve	weak	-ve
CT-Cyt	weak	weak	-ve	very weak	-ve
Pap-Cyt	weak	weak	-ve	-ve	-ve

Cytochrome b_{561}	Cyt
Carboxymethylated cyt. b_{561}	CM-Cyt
CnBr-cleaved cyt. b_{561}	CnBr-Cyt
Chymotrypsin-cleaved cyt b_{561}	CT-Cyt
Papain-cleaved cyt b_{561}	Pap-Cyt

6.02.04.: Preparation of Antiserum to Cyanogen Bromide Fragments of Cytochrome b_{561}

Cytochrome b_{561} contains 7 methionine residues and therefore cyanogen bromide cleavage of cytochrome b_{561} should produce 8 polypeptide fragments, each with different antigenic determinants. Cytochrome b_{561} was treated with cyanogen bromide as described in Section 2.02.08. and the resulting fragments used to immunize a New Zealand white rabbit (Section 2.05.01.). Unfortunately the fragments produced by cyanogen bromide cleavage of cytochrome b_{561} were not able to be characterized on SDS-polyacrylamide gels because of their small size.

6.02.05.: Testing the Antisera Produced against Various Modified Forms of Cytochrome b_{561}

The ability of the various resulting antisera to recognize the various chemically and enzymatically modified forms of cytochrome b_{561} was tested using the immune replica technique (Section 2.04.05.). The results are tabulated in Table 6.02.01.. The best antiserum was that produced against reduced and S-carboxymethylated cytochrome b_{561}. As shown in Fig.6.02.02., this antiserum reacts strongly with cytochrome b_{561}, S-carboxymethylated cytochrome b_{561} and chymotrypsin-cleaved cytochrome b_{561}. The fact that this antiserum recognizes the chymotrypsin-cleaved cytochrome b_{561} was particularly interesting since the original antiserum against cytochrome b_{561} did not recognize this polypeptide. This supports

the idea that cytochrome b_{561} does contain disulphide bonds and also that new antigenic determinants are revealed by irreversibly reducing the protein. The other three antisera reacted only weakly with all forms of cytochrome b_{561}. The chymotrypsin-cleaved and papain-cleaved cytochromes would be expected to be hydrophobic and therefore possibly not very good immunogens. The fragments produced by cyanogen bromide treatment of cytochrome b_{561} may have been too small to elicit an immune response.

6.02.06.: Immunoprecipitation of Cytochrome b_{561} from Translation Reactions

The antisera described above were all tested for their ability to immunoprecipitate the precursor to cytochrome b_{561} from translation reactions performed in either reticulocyte lysate or wheatgerm extract. However, none of the antisera precipitated any detectable translation products. It has proved difficult to immunoprecipitate the polypeptide precursors to hydrophobic proteins such as ovalbumin, due to their tendency to aggregate. This difficulty has been overcome by having purified immunoglobulin G present at the start of the translation reaction (Palmiter *et al.*, 1977). Immunoglobulin G was prepared from antisera to cytochrome b_{561} and S-carboxymethylated cytochrome b_{561} as described in Section 2.05.02.. Pure immunoglobulin G (5µg) was then added at the beginning of the translation reaction and subsequently isolated using *S. aureus* cells as in the normal immune-precipitation procedure (Section 2.03.12.). This method also failed to immunoprecipitate any detectable translation products.

6.02.07.: Possible Explanations for the Failure to Immunoprecipitate a Precursor to Cytochrome b_{561} from a Translation of Adrenal Medullary messenger RNA

There are several possible explanations for the failure to immunoprecipitate the precursor to the second most abundant chromaffin granule membrane protein, cytochrome b_{561}, from translation reactions. It is possible that none of the antisera produced against cytochrome b_{561} are capable of recognizing or precipitating the immediate precursor of in vitro translation. Alternatively the messenger RNA species encoding cytochrome b_{561} may be a weakly initiating species and may not be translated efficiently with the result that not enough radiolabelled amino acid would be incorporated into the polypeptide for it to be detected by fluorography. The other and most likely alternative is that very few copies of the messenger RNA are present in the RNA preparation. This may be due to the adrenal medulla not having to synthesize much cytochrome b_{561}. This in turn could be due to the fact that after exocytosis, the chromaffin granule membranes are recycled (Section 1.11.) thereby reducing the medullary cells' need to resynthesize granule membrane proteins. This is probably also the reason for the failure to identify the precursor to membrane bound dopamine β-hydroxylase. In conclusion, no precursors to the two major chromaffin granule membrane proteins have been immunoprecipitated from translation reactions of adrenal medullary messenger RNA performed in either reticulocyte lysate or the wheatgerm S-30 translation system.

CHAPTER 7

IDENTIFICATION OF CALMODULIN FROM TRANSLATION PRODUCTS

RESULTS AND DISCUSSION

FIGURE 7.01.

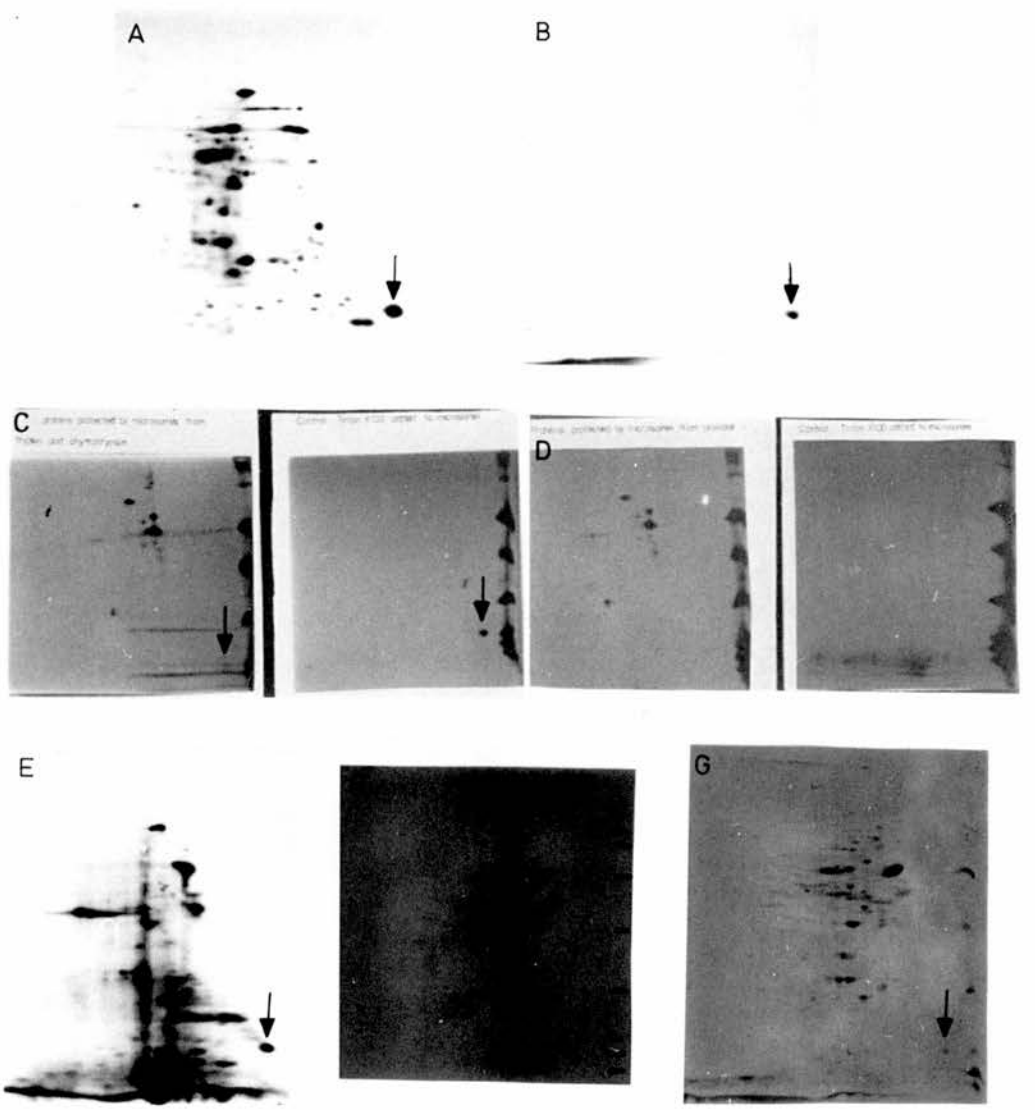


FIGURE 7.01.: Legend

A. Fluorograph of 2-dimensional gel electrophoretogram of translation products (Section 2.03.04.)

B. Coomassie-stained 2-dimensional gel electrophoretogram of 10ug of bovine brain calmodulin

C. Post-Translational Proteolysis Assay (Section 2.03.09.) using chymotrypsin and trypsin; control performed in the presence of Triton X-100

D. Post-Translational Proteolysis Assay (Section 2.03.09.) using pronase; control performed in the presence of Triton X-100

E. Fluorograph of 2-dimensional gel electrophoretogram of polypeptides synthesized when reticulocyte lysate was supplemented with adrenal medullary bound polysomes (Section 4.07.02.)

F. Fluorograph of the polypeptides synthesized by chromaffin cells during a 15 minute incubation in the presence of ^{35}S-methionine (Section 5.02.01.)

G. Coomassie-stained 2-dimensional gel electrophoretogram of chromaffin cellular proteins

7.01.: Identification of Calmodulin from the Translation Products when Adrenal Medullary messenger RNA is translated in Reticulocyte Lysate

One of the polypeptides synthesized during the translation of adrenal medullary messenger RNA in reticulocyte lysate drew attention to itself for several reasons:-

1. it was a major translation product of apparently low molecular weight and isoelectric point, into which approximately 1% of the TCA-precipitable ^{35}S-methionine was incorporated (Fig.7.01.A)
2. it was resistant to proteolysis by chymotrypsin and trypsin (Fig. 7.01.C) but sensitive to digestion with pronase (Fig. 7.01.D), in the control experiments of the post-translational proteolysis assay for functional dog pancreas microsomes which were performed in the presence of 1% Triton X-100 (Section 4.06.02.)
3. it was synthesized when adrenal medullary bound polysomes were run-off in reticulocyte lysate (Section 4.07.02. and Fig.7.01.E.) and also during *in vivo* cellular protein synthesis (Section 5.02. and Fig. 7.01.F). It was also identifiable on Coomassie blue-stained 2-dimensional electrophoretograms of total adrenal cellular proteins (Fig. 7.01.G).

There exists a family of low molecular weight, acidic Ca^{2+}-binding proteins including troponin C, brain S-100, parvalbumin, intestinal Ca^{2+}-binding protein and calmodulin (Baba *et al.*, 1984). Calmodulin is the best characterized of these proteins. It is present in all eukaryotic cells, has a molecular weight of 16,700, an

isoelectric point of 3.9-4.3 and is involved in the regulation of glycogen metabolism, cyclic nucleotide metabolism, protein phosphorylation, microtubule assembly, Ca^{2+} flux, exocytosis and endocytosis (reviewed in Klee et al., 1980; Means et al., 1982). The amino acid sequences of calmodulins from various sources have been determined and it has been found to be highly conserved throughout evolution.

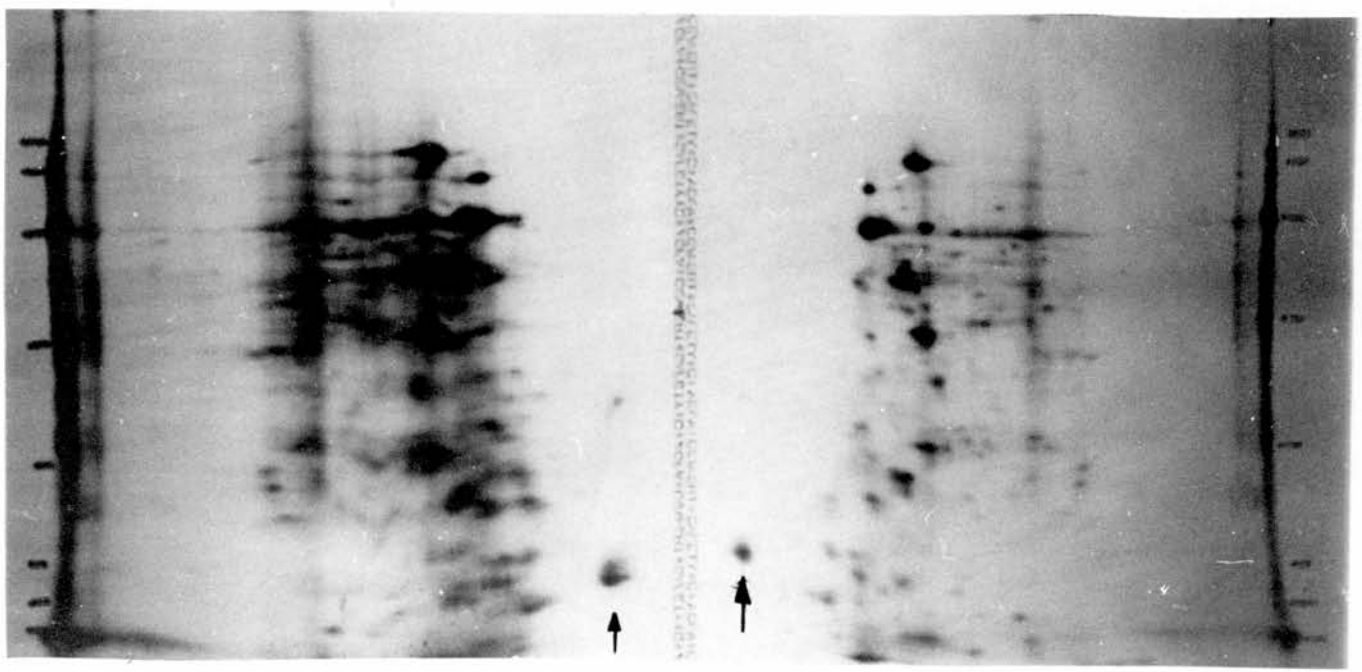
As discussed in Section 1.12., Ca^{2+} plays a key role in the process of secretion (Douglas, 1968), and calmodulin, the major Ca^{2+}-binding protein in non-muscle cells, has been shown by using antibodies, to be directly involved with exocytosis from the chromaffin cell (Kenigsberg and Trifaro, 1985). Several calmodulin-binding proteins have been identified as components of chromaffin granule membranes (Section 1.12.). Calmodulin has been calculated by radioimmunoassay, to comprise approximately 0.04% of the total adrenal medullary protein (Hikita et al., 1984). It was therefore possible that the translation product of similar molecular weight and isoelectric point to calmodulin was actually calmodulin.

7.02.: The Low Molecular Weight Acidic Translation Product Co-migrates with Calmodulin on 2-Dimensional Gels

A 2-dimensional gel separation of calmodulin is shown in Fig.7.01.B.. The radiolabelled translation products from adrenal medullary messenger RNA were co-electrophoresed on a 2-dimensional gel with 10µg of commercial bovine brain calmodulin. The resulting gel was stained with Coomassie blue prior to fluorography. When the

Fig.:-
7.02.

Does small acidic protein from translation products exhibit Ca^{2+} binding properties characteristic of Calmodulin?



Translation products run in the presence of Ca^{2+}

Translation products run in the presence of EGTA (mirror image of fluorograph)

resulting fluorograph was positioned exactly on top of the Coomassie blue-stained gel, the small acidic translation product was observed to co-migrate exactly with bovine brain calmodulin.

7.03.: The Mobility of the Low Molecular Weight Translation Product is Sensitive to Ca^{2+} on SDS-polyacrylamide gels

In the absence of Ca^{2+}, 40% of the polypeptide chain of calmodulin exists in an α-helical conformation. However, in the presence of Ca^{2+}, this is increased to greater than 50% (Dedman et al., 1977). The mobility of calmodulin during SDS-polyacrylamide gel electrophoresis is enhanced in the presence of Ca^{2+} (Grab et al., 1979; Burgess et al., 1980). This phenomenon can be used as one of the criteria for identifying calmodulin from a mixture of proteins. The other Ca^{2+} binding proteins do not exhibit such a mobility shift in the presence of Ca^{2+} (Burgess et al., 1980).

Adrenal medullary messenger RNA translation products, together with 10ug of commercial bovine brain calmodulin, were subjected to isoelectric focusing (Section 2.04.04.) in the presence of either 0.1mM EGTA or 0.1mM Ca^{2+}. After focusing, the respective tube gels were soaked in tube gel soaking buffer (Section 2.04.04.) either in the presence of 0.1mM EGTA or 0.1mM Ca^{2+} before SDS-polyacrylamide gel electrophoresis on 8-15%(w/v) acrylamide gels either made with the usual EDTA-containing gel buffers (Section 2.04.01.) in the case of the tube gel which had been exposed to EGTA, or with gel buffers without EDTA for the tube gel which had been exposed to 0.1mM Ca^{2+}. The resulting 2-dimensional gels were Coomassie blue-stained prior to

FIGURE 7.03.: Fluorograph of the polypeptides produced by limited proteolytic digestion (with trypsin) of calmodulin, excised from 2-dimensional gel separations of translation products. Digestion was performed in the presence of 100ug of bovine brain calmodulin.

Lane 1 : control; no protease

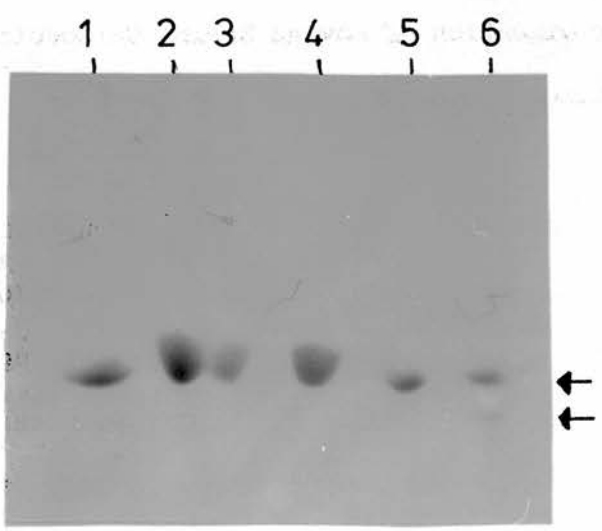
Lane 2 : 0.02µg of trypsin

Lane 3 : 0.2µg of trypsin

Lane 4 : 2µg of trypsin

Lane 5 : 10µg of trypsin

Lane 6 : 20µg of trypsin



fluorography. As shown in Fig. 7.02., calmodulin and the low molecular weight translation product co-migrate with an apparent molecular weight of about 21,000 when electrophoresed in the presence of EGTA whereas, in the presence of Ca^{2+}, they co-migrate with an apparent molecular weight of about 17,000. This shift in the electrophoretic mobility of the small translation product in the presence of EGTA suggests that this translation product is calmodulin. This premise has been used previously to identify calmodulin from spinach leaf translation products (Van Eldik et al., 1980). It is interesting to note that this newly-synthesized calmodulin from spinach leaf translation products did not contain a trimethyl-lysine residue but still exhibited Ca^{2+} sensitivity (Van Eldik et al., 1980).

7.04.: Tryptic Digestion of Bovine Brain Calmodulin and the Small Acidic Translation Product

Adrenal medullary mesenger RNA translation products were separated by 2-dimensional gel electrophoresis along with 50μg of commercial bovine brain calmodulin. The resulting gels were stained with Coomassie blue and the easily identifiable calmodulin spots were excised from the gel. The excised gel segments were then digested with various concentrations of trypsin during electrophoresis as described in Section 2.04.03.. The resulting 15% SDS-polyacrylamide gel was stained with Coomassie blue prior to fluorography. The resulting radiolabelled proteolytic fragments seen by fluorography (Fig. 7.03.), corresponded exactly with the Coomassie-stained fragments, although it is clear that higher ratios of trypsin to

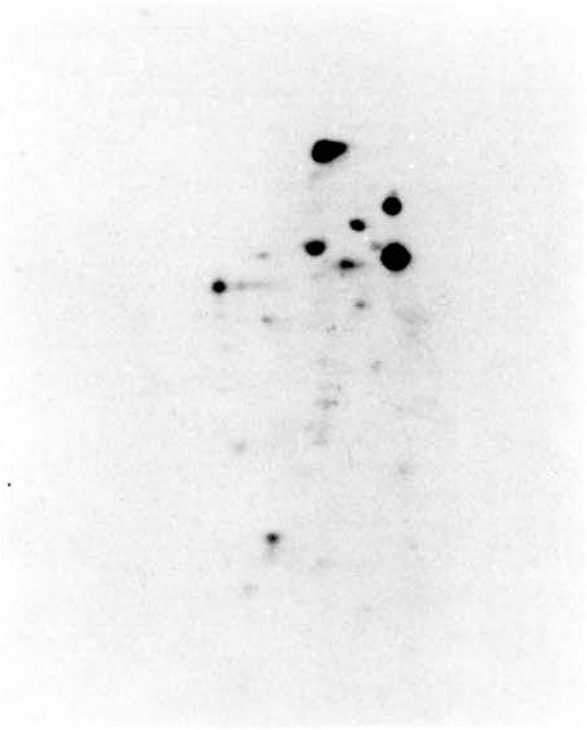


Fig.: 7.04.A. Polypeptides associated with the microsomal pellet

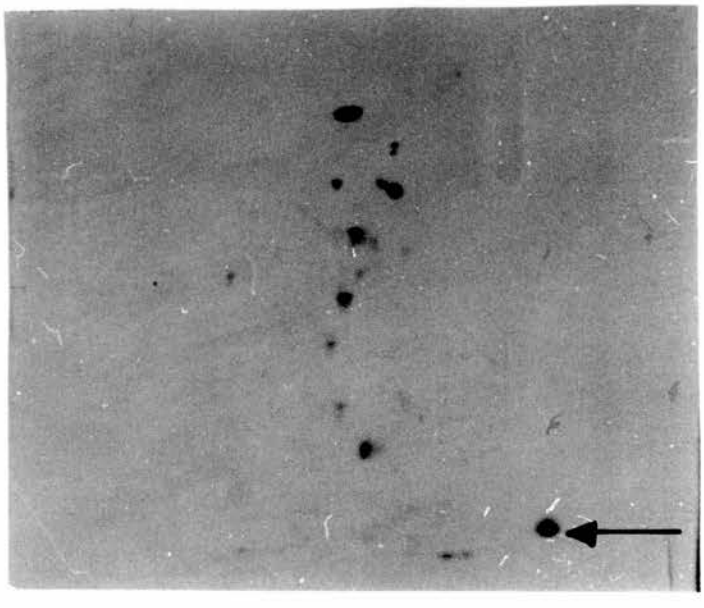


Fig.: 7.04.B. Polypeptides remaining in the supernatant after centrifugation to remove the microsomes.

calmodulin would be required for complete digestion. In the absence of EGTA, digestion of calmodulin with trypsin results mainly in the cleavage of the peptide bond between lysine 77 and aspartate 78 (Walsh and Stevens, 1977). The result obtained does therefore lend further support to the identity of the small acidic translation product being calmodulin.

7.05.: Calmodulin from Adrenal Medullary Messenger RNA Translation Products: Summary and Conclusions

The evidence presented above suggests that the small acidic translation product (Fig. 7.01.A), is calmodulin. This polypeptide is not translocated across microsomal membranes since, when the microsomes from a translation performed in the presence of dog pancreas microsomes (Section 4.06.01.) were isolated by centrifugation (Section 2.03.08.), and the radiolabelled polypeptides associated with the microsomal pellet examined by 2-dimensional gel electrophoresis, the small acidic polypeptide was not present (Fig. 7.04.A.). It remained instead in the supernatant (Fig.7.04.B.). This was also supported by results obtained from the post-translational proteolysis assay for functional dog pancreas microsomes (Section 4.06.02.). In this experiment the small acidic spot appeared, at first, to be protected by the microsomes from digestion with trypsin and chymotrypsin (Fig. 7.01.C). However, even in the control experiment, where the microsomal membranes were disrupted with Triton X-100 before the addition of trypsin and chymotrypsin, the small acidic polypeptide remained resistant to digestion (Fig.7.01.C). This experiment was performed in the

presence of 8mM Ca^{2+} to prevent autolysis of trypsin (Section 2.03.09.). In the presence of Ca^{2+}, very high concentrations of trypsin are required to digest calmodulin (Walsh and Stevens, 1977). This would account for the apparent resistance of the small acidic translation product to trypsin and chymotrypsin. Consequently, pronase, a less specific protease, was used in the post-translational proteolysis assay and the small acidic polypeptide was found not to be protected by the microsomes (Fig. 7.01.D).

The observation that the small acidic translation product was synthesized when reticulocyte lysate was supplemented with adrenal medullary bound polysomes (Fig.7.01E) was rather surprising, although this could simply be due to contamination of the bound polysome preparation with free polysomes. Calmodulin is a cytoplasmic protein, although it is bound to different membrane fractions in the adrenal medulla to varying extents depending on the presence or absence of Ca^{2+} (Hikita et al., 1984). Calmodulin has, however, previously been characterized, together with several cytosolic enzyme proteins, including creatine Kinase, pyruvate kinase and neuron-specific enolase, as being synthesized on both the membrane-bound and free polysomes of rat forebrain (Hall et al., 1984). This finding was proposed to be significant for axonal transport since these proteins are all major components of the slow component of axonally transported proteins. It would be necessary to rule out the possibility of contaminating free polysomes in the preparation of adrenal medullary bound polysomes before speculating on the significance of adrenal medullary calmodulin being synthesized on membrane-bound polysomes.

Table 7.01.

Calmodulin as a Percentage of Total Protein in Various Tissues

Electroplax of *Electrophorus electricas*	2.5%	Means and Chafouleas., 1982
Nerve ending fraction from bovine pituitary	1%	Sheaves *et al.*, 1980
Bovine posterior pituitary	0.5%	Sheaves *et al.*, 1980
Chicken gizzard	0.4%	Head *et al.*, 1980
Bovine brain	0.5%	Watterson *et al.*, 1976
Rat testis	0.016%	Dedman *et al.*, 1977
Bovine heart	0.014%	Teo *et al.*, 1973
Bovine adrenal medulla	0.04%	Hikita *et al.*, 1984
Bovine adrenal medulla	0.01-0.02%	Kuo and Coffee, 1976
Bovine adrenal medulla	0.2%	Tirrell and Coffee, 1983

Calmodulin constitutes between 0.01%-1% of the total protein in eukaryotic cells (Means and Chafouleas, 1982), although in the electroplax of the eel Electrophorus electricus calmodulin represents between 2%-5% of the total protein. Table 7.01. lists calmodulin as a percentage of total protein from various tissues. It is not surprising to find calmodulin among the adrenal medullary messenger RNA translation products since, as mentioned above, it has been calculated to be 0.04% of the total adrenal medullary protein (Hikita et al., 1984) and has been shown to be directly involved in exocytosis from the adrenal medulla using anti calmodulin immunoglobulins (Kenigsberg and Trifaro, 1985). It is surprising that as much as 1% of the ^{35}S-methionine incorporated into TCA-precipitable material, as determined by eluting the radioactivity from the excised gel segment (Section 2.05.04.), is incorporated into calmodulin. This is almost 25% as much as the most abundant adrenal medullary secretory protein, chromogranin A (Section 5.01.02.). However, little quantitative data can be extracted from the in vitro translation results since, as discussed previously (Section 4.09.), it is not possible to correlate the number of molecules of a particular messenger RNA with the amount of a particular polypeptide synthesized, since large quantities of polypeptide may be synthesized simply because the messenger RNA encoding the polypeptide may be a relatively strong initiator. The in vitro translation in reticulocyte lysate of the total poly A^+ isolated from the electroplax resulted in about 10% of the ^{35}S-methionine incorporated into TCA-precipitable material being incorporated into calmodulin (Munjaal et al., 1980). This confirms that the percentage of calmodulin synthesized during in vitro

translation is artificially high. Calmodulin also contains a relatively high proportion of methionine residues, 9 from a total of 148 amino acids (Watterson *et al.*, 1980).

During *in vivo* cellular synthesis calmodulin is still a relatively major product. About 0.45% of the ^{35}S-methionine which is incorporated into TCA-precipitable material, is incorporated into calmodulin. This is still considerably higher than the percentage of calmodulin calculated to be present in the adrenal medulla (Hikita *et al.*, 1984), but this may be due to calmodulin having a high turnover rate or to the relatively high proportion of methionine residues in the polypeptide. It may also be due to an increased synthesis of calmodulin in the newly-isolated cells.

In conclusion, a small acidic adrenal medullary messenger RNA translation product has been tentatively identified as calmodulin from the fact that it co-migrated with commercial bovine brain calmodulin, its mobility exhibited Ca^{2+}-sensitivity during SDS-polyacrylamide gel electrophoresis and its tryptic digest fragments were identical to those of commercial calmodulin. The extensive incorporation of ^{35}S-methionine into this polypeptide during *in vitro* translation of adrenal medullary messenger RNA may be due to the calmodulin messenger RNA being a strong initiator.

CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

CHAPTER 8.: General Discussion and Conclusions

The studies described in this thesis give some insight into the events occurring during the biosynthesis of the major protein of the chromaffin granule matrix, chromogranin A (Chapter 5) and also some preliminary information about the biosynthesis of dopamine B-hydroxylase, apparently relating to the soluble form of this enzyme (Chapter 6). The primary polypeptide precursors to these proteins have been identified, and some aspects of their post-translational modification have been clarified. In particular, the derivation of the chromogranin A family of polypeptides from a common precursor by the action of intragranular proteases could be inferred from the results. These studies have not succeeded in identifying the polypeptide precursors to the major chromaffin granule membrane proteins, dopamine β-hydroxylase and cytochrome b_{561}. There are several possible explanations for this failure:

1. The messenger RNA encoding these proteins may have been destroyed during the preparation of the RNA. Selective degradation of the messenger RNA's encoding the two major chromaffin granule proteins does however, seem an unlikely coincidence.

2. It is possible that the antisera raised against the "mature" forms of dopamine β-hydroxylase and cytochrome b_{561} simply did not recognise the polypeptide precursors to these proteins. However, this seems unlikely. In the case of dopamine β-hydroxylase, the antiserum is unlikely to immunoprecipitate the precursor to the soluble form but not to that of the membrane-bound form of the enzyme, when both forms of the enzyme are immunologically

indistinguishable. Furthermore, the antiserum also reacts with chemically deglycosylated dopamine β-hydroxylase (Apps et al., 1985), so is not directed solely against the oligosaccharide chains of the glycoprotein. In the case of cytochrome b_{561}, antisera to various modified forms of the protein were produced in case the original antiserum to the mature protein failed to recognise the precursor (Section 6.02.); these antisera all failed to immunoprecipitate a precursor.

3. Another more likely explanation is that not enough radiolabelled amino acid was incorporated into the precursors to membrane-bound dopamine β-hydroxylase or cytochrome b_{561} to allow them to be detected by fluorography (Section 4.09.). This may have been due to inefficient translation of the messenger RNA molecules encoding these proteins (Section 4.09.). Alternatively there simply may have been low levels of the messenger RNA molecules encoding these proteins.

There are two aspects of the biology of chromaffin cells which suggest that there might be only low levels of messenger RNA molecules encoding chromaffin granule membrane proteins:-

1. chromaffin granule membrane recycling
2. storage of large quantities of chromaffin granules which have a low turnover.

As discussed in Section 1.05.01., exocytosis is invariably followed by endocytosis, the retrieval of the secretory granule membrane (reviewed by Farquhar, 1983). Reutilization of the chromaffin granule membrane proteins obviously reduces the cells' requirement to synthesize those proteins. It has been estimated that

chromaffin granule membrane proteins are reutilized about five times before being returned to lysosomes for degradation (Winkler, 1977). In contrast, the chromaffin granule matrix proteins which are lost by exocytosis are having to be constantly replenished. The intracellular levels of messenger RNA encoding chromaffin granule matrix proteins would therefore be expected to be much higher than those encoding chromaffin granule membrane proteins.

Chromaffin cells store large quantities of chromaffin granules. It has been estimated that each bovine chromaffin cell contains 34,000 granules (Phillips, 1982). In experiments performed to demonstrate exocytosis from the adrenal medulla, secretion is usually induced by massive over-stimulation of the cells, resulting in the release of up to 70% of the total catecholamine pool. The release of catecholamine from cat adrenal gland perfused in situ, has been correlated with the release of acetylcholine from the splanchnic nerve (Collier et al., 1984). The release of one quantum of acetylcholine resulted in the release of one quantum of catecholamine. It has been calculated that under physiological conditions, each splanchnic nerve impulse would result in the release of the contents of 0.4 granules per bovine chromaffin cell on average (reviewed by Phillips, 1982). The turnover of granules may therefore be quite low. The half-life of the catecholamine pool has been estimated as greater than one week (reviewed by Winkler, 1977). This is in contrast to the high turnover of the contents of pancreatic cell zymogen granules which have a half-life of 10-15 minutes (Palade, 1975).

The slow turnover of chromaffin granules in combination with granule membrane recycling supports the suggestion that the cell would require to synthesize only small amounts of granule membrane proteins and therefore only a small proportion of the total messenger RNA molecules would be likely to encode such proteins.

As discussed previously (Section 1.01.), the chromaffin granules of the bovine adrenal medulla are the best characterized secretory organelles due to the ease with which large quantities of granules can be prepared. As such, chromaffin granules appeared to offer a particularly good model with which to study the biogenesis of secretory organelles. However, as also discussed in Section 1.01., central to the problem of secretory granule biogenesis, is the origin of the secretory granule membrane. In view of the fact that in these studies it has proved impossible to characterize the polypeptide precursors to the two major chromaffin granule membrane proteins, the adrenal medulla would not appear to provide an attractive model system with which to study the biogenesis and assembly of complete secretory organelles. Instead, at present the adrenal medulla provides the ideal system with which to study many aspects of "mature" secretory organelles and hormone storage.

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Biosynthetic relationship between the major matrix proteins of adrenal chromaffin granules

Lynn Kilpatrick, Fiona Gavine, David Apps and John Phillips

Department of Biochemistry, University Medical School, George Square, Edinburgh EH8 9XD, Scotland

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The matrix of the chromaffin granule contains a family of acidic proteins, collectively known as the chromogranins. It has been suggested that this family results from protease action on the major component, chromogranin A. Evidence for this has now been obtained from in vitro translation of adrenal medullary messenger RNA and immunoprecipitation of translation products using an antiserum directed against chromogranin A, but which also recognises other chromogranins.

Chromogranin *Chromaffin* *Messenger RNA* *Antibody*

1. INTRODUCTION

Chromaffin granules, the catecholamine storage organelles of the adrenal medulla, contain high concentrations of several low-M_r substances, including the catecholamines (noradrenaline and adrenaline), nucleotides (particularly ATP), calcium and magnesium ions, and ascorbate [1]. Protein components of the granule matrix include a soluble form of dopamine-β-hydroxylase, which is involved in catecholamine biosynthesis and occurs also as a granule membrane protein, enkephalins and their high-M_r precursors, and a family of acidic proteins, the chromogranins [1,2]. The major chromogranin, comprising 50% of matrix protein, has been named chromogranin A [3,4].

Chromogranin A has an M_r of about 70000 and NMR and ORD studies suggest that it exists mainly as a random coil interrupted by only a small segment of α-helix [5]. Amino acid analysis of chromogranin A and of the remaining chromogranins reveals a similar composition [6]. This in itself suggests a relationship between the chromogranins, and their isoelectric points are very similar [7]. This raises an important question about the biogenesis of the chromogranins: are they all encoded by a single gene which is transcribed into a single mRNA, giving rise to a precursor which generates a series of proteins by post-translational processing? Alternatively, is each member of the chromogranin family encoded by a distinct messenger RNA, transcribed from a family of genes and translated separately?

It has been suggested that the function of the chromogranins within the chromaffin granule is to stabilize a storage complex involving catecholamines and ATP. Recently, however, structural similarities between chromogranin A and secretory proteins of other hormone-storing granules have been detected by using immunological methods [8,9]. This raises the possibility that this type of protein plays a general role in storage or secretory processes and that similar proteins may be found in other secretory tissues.

2. MATERIALS AND METHODS

Chromogranin A was purified from a lysate of bovine chromaffin granules by chromatography on DEAE-cellulose [10], followed by passage through concanavalin A–Sepharose to remove dopamine-β-hydroxylase. The resulting mixture of chromogranins was resolved by electrophoresis on a 10% polyacrylamide slab gel in the presence of sodium dodecyl sulphate, briefly stained, and the

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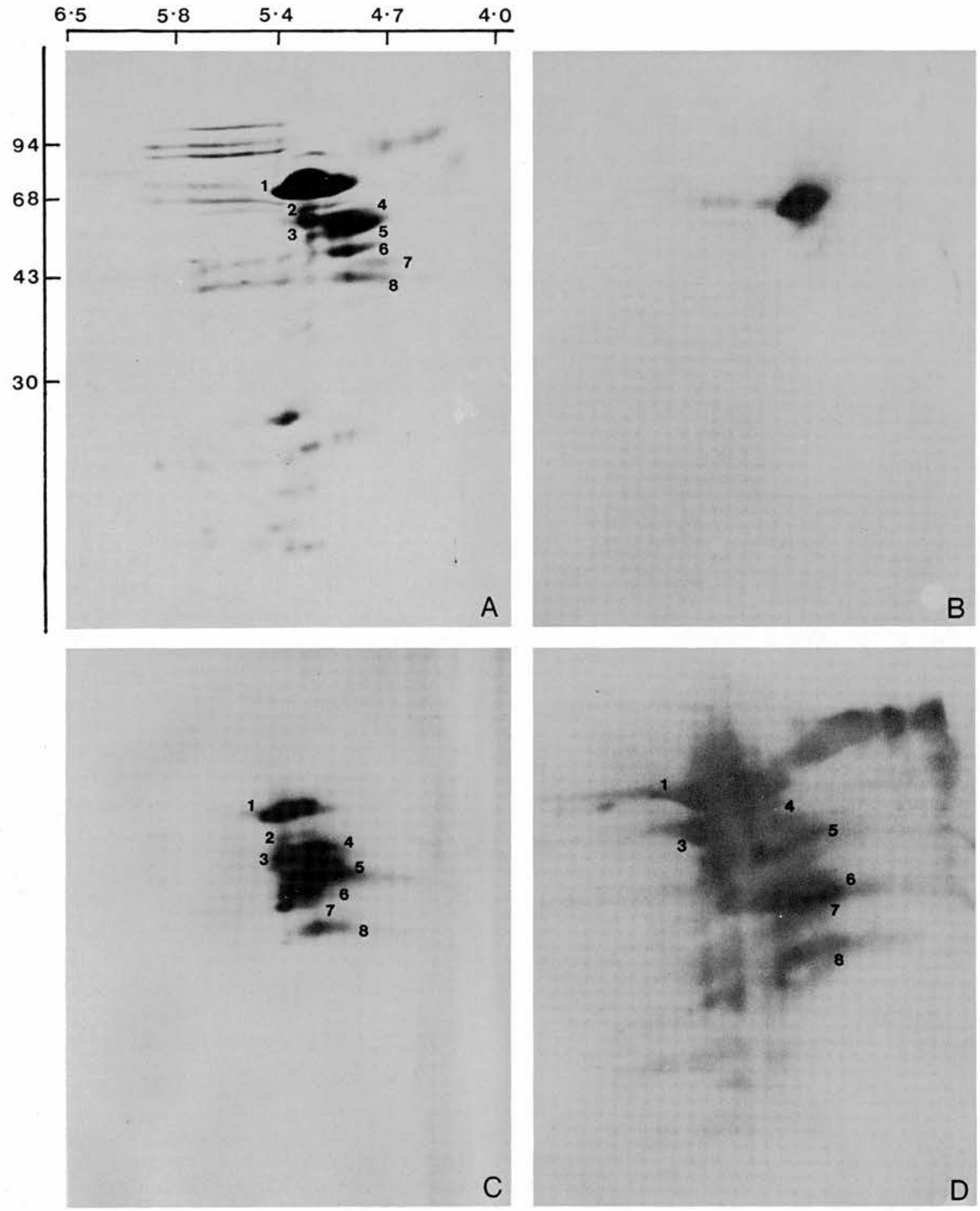
6·5
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43
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8
A
B
C
D

band of chromogranin A cut out. Pure chromogranin A was obtained by electroelution: this material was used to immunise New Zealand white female rabbits.

Radioiodinated chromogranins were prepared by iodination of total chromaffin granule lysate or of the mixed chromogranin peak from the DEAE-cellulose column, using the chloramine-T method.

Total cellular RNA was prepared from bovine adrenal medullae by guanidinium thiocyanate extraction [11]. Poly-adenylated RNA was subsequently purified by affinity chromatography on oligothymidilic acid cellulose [12] and translated in a message-dependent reticulocyte lysate [13] in the presence of [^{35}S]methionine (1.85×10^7 Bq/ml).

Immunoprecipitation of the translation product recognised by antiserum against chromogranin A was performed essentially as in [14].

Two-dimensional gel electrophoresis [15] was performed using Bio-Rad ampholines with a pH range of 4–6 in the first dimension and 8–15% SDS polyacrylamide gels in the second dimension.

Immune replicas [6] were made by electrophoretic transfer of proteins, separated by two-dimensional gel electrophoresis, onto nitrocellulose sheets. After thorough washing in a bovine serum albumin-containing buffer, the replica was decorated with antiserum to chromogranin A; after further washing, iodinated protein A (10^4 Bq/ml) was used to label the attached antibodies. The washed and dried nitrocellulose sheet was then autoradiographed.

3. RESULTS

3.1. *Cross-reaction of antiserum to chromogranin A with other chromogranins*

The result of two-dimensional gel electrophoretic separation of chromaffin granule lysate components, followed by staining with Coomassie blue, is shown in fig.1A. Chromogranin A is the dominant component with a p*I* in the range 4.5–4.9 and apparent M_r about 70000. The other chromogranins appear as spots of similar isoelectric point but decreasing M_r.

Two-dimensional electrophoresis of pure chromogranin A, followed by electroblotting and immune replication using anti-chromogranin A serum, demonstrates the purity of the chromogranin A used to raise the antiserum (fig.1B). However, an immune replica of total granule lysate, using the same serum, reveals that this antiserum can recognise other chromogranins present in the lysate in addition to chromogranin A (fig.1C). The number of cross-reacting lysate components revealed varies considerably with the amount of protein loaded. A light loading of protein (fig.1C) reveals a small, well-defined group of proteins with similar p*I* values, the largest of which is chromogranin A. These correspond to most of the major spots in fig.1A. On the other hand, a larger group of proteins with a greater range of isoelectric point and M_r are shown to be capable of reaction with anti-chromogranin A serum when a larger amount of protein is loaded (fig.1D).

Immunoprecipitation studies using ^{125}I-labelled granule lysate confirm that several chromogranins are recognised by antiserum to chromogranin A. Fig.2A shows an autoradiograph of a two-dimensional separation of the total ^{125}I-lysate components. The components of this unfractionated granule lysate which are immunoprecipitated by anti-chromogranin A serum are shown in fig.2B. In agreement with the results of fig.1C, this includes most of the chromogranin group (M_r in the range 70000–40000), but only traces of material of greater M_r than chromogranin A itself.

3.2. *Immunoprecipitation of in vitro translation products*

Anti-chromogranin A serum was added directly to the radiolabelled translation products from

←

Fig.1. Two-dimensional electrophoresis of chromaffin-granule lysate proteins. (A) Total lysate, stained with Coomassie blue (gel loaded with 50 μg protein). The upper scale gives the pH calibration; M_r markers were phosphorylase (94000), bovine serum albumin (68000), ovalbumin (40000) and carbonic anhydrase (30000). Bands are numbered on the gel to aid identification in other figures; the numbers are not intended as a new classification of chromogranins. (B–D) Autoradiographs of immune replicas using anti-chromogranin A serum and ^{125}I-protein A: (B) purified chromogranin A (10 μg); (C) total lysate (2 μg); (D) total lysate (20 μg). The immune replica technique identifies material on the gel that cross-reacts with the serum; as used here, it does not give quantitative information about the amount of such material present.

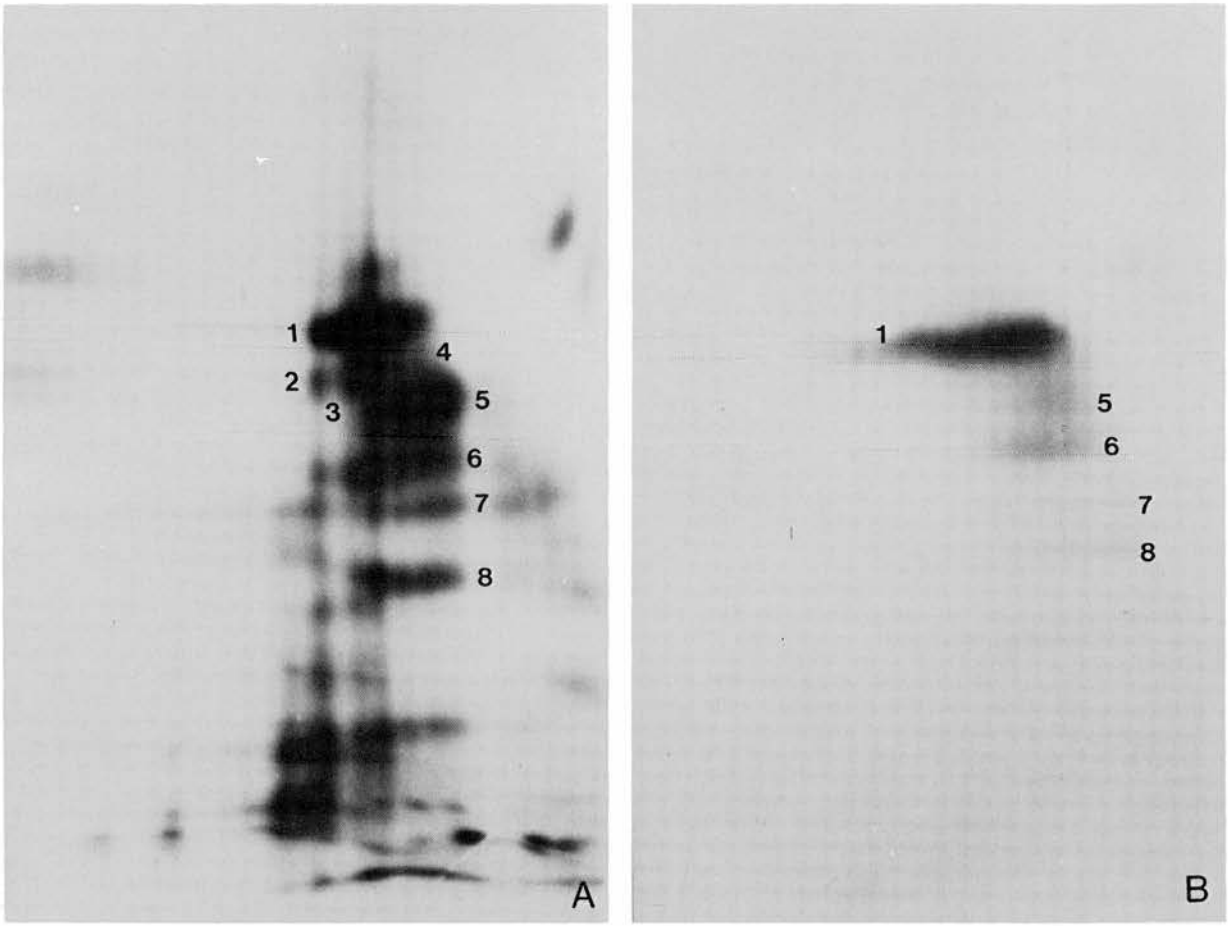

Fig.2. Two-dimensional electrophoretic separation of radioiodinated chromaffin-granule lysate proteins. (A) Autoradiograph of total lysate (compare with Coomassie blue-stained gel, fig.1A; the amount of ^{125}I incorporated into the polypeptides does not correlate closely with their staining intensity); (B) autoradiograph of proteins precipitated with anti-chromogranin A serum.

adrenal medullary mRNA, and immune complexes were precipitated by the addition of formalin-fixed *Staphylococcus aureus* cells. The immunoprecipitated translation product was eluted from the cells and 30 μg unlabelled lysate proteins added as carrier. A two-dimensional electrophoretogram of this mix was stained with Coomassie blue to reveal the position of the lysate proteins (fig.3A) and then autoradiographed to reveal the position of the ^{35}S-labelled translation product(s) recognised by the antiserum (fig.3B).

The two adjoining spots on the autoradiograph comigrate with the low-M_r end of the broad chromogranin A spot, with a p*I* slightly shifted towards the acidic side. No other translation products, either larger or smaller, were precipitated by the anti-chromogranin A serum.

It is interesting to note the effect of adding non-radioactive lysate proteins to the immunoprecipitated translation product on the pattern of radiolabelled spots obtained after autoradiography. A two-dimensional separation of the same immunoprecipitated translation products, but in the absence of lysate proteins as carrier, resulted in 4 distinct small spots in the same position as the two in fig.3B (see fig.3C). Presumably the higher loading of protein affected the resolution of the electrofocussing.

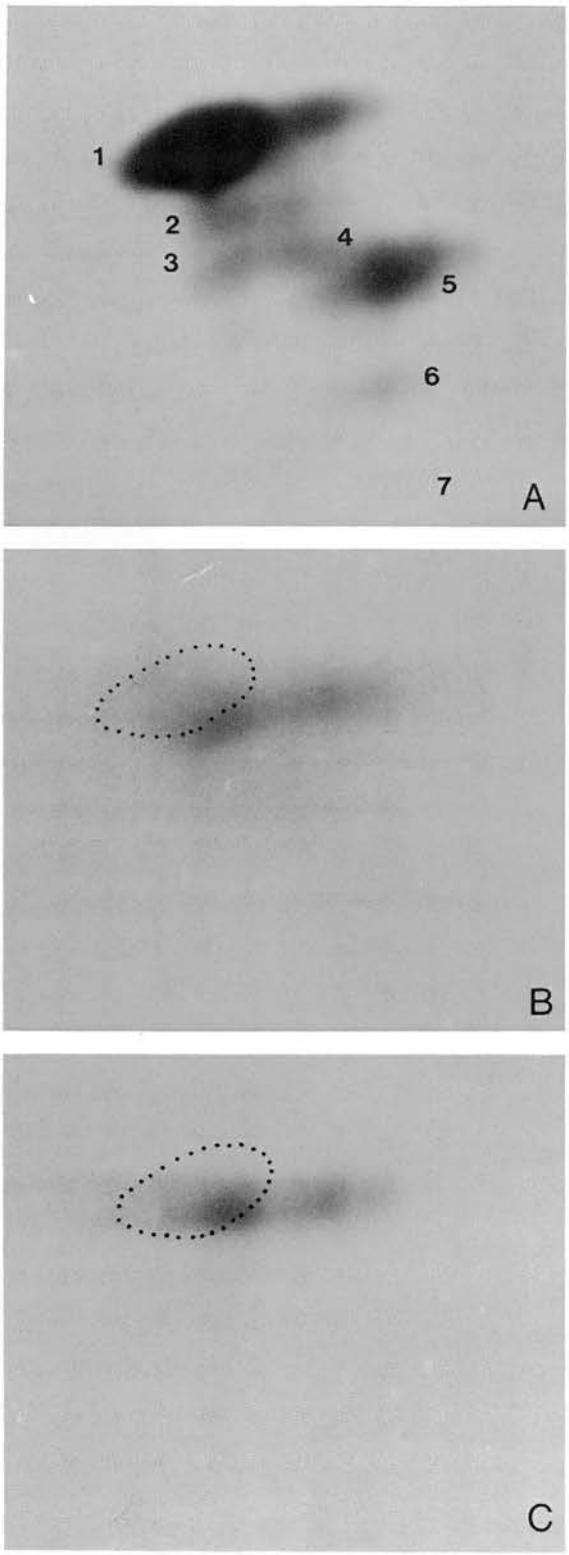

Fig.3. Two-dimensional electrophoretic separation of chromogranins synthesised by translation in vitro and precipitated with anti-chromogranin A serum. Only the region of the gel around the chromogranin A spot (fig.1B) is shown; the rest of each gel was blank. (A) Gel stained with Coomassie blue; lysate proteins were added as carrier to the immunoprecipitate; (B) autoradiograph of gel shown in (A), showing ^{35}S-labelled translation products; (C) autoradiograph of a similar gel run without carrier. In (B) and (C) the position of the major Coomassie blue-stained protein has been indicated.

4. DISCUSSION

The slight differences in M_r and isoelectric point between mature chromogranin A, and the product of in vitro translation, are presumably the result of post-translational processing. Chromogranin A is a glycoprotein containing about 4% carbohydrate with terminal sialic acid residues [4,17], so that the mature product would be expected to be of higher M_r and lower isoelectric point than the immediate product of translation. In fact, its isoelectric point is somewhat higher (fig.3B), suggesting that other modifications, such as proteolysis, may also occur during maturation in vivo.

As shown in fig.1A, the chromaffin granule lysate contains several minor components of higher M_r than chromogranin A itself, including one of somewhat lower isoelectric point. Using a heavy loading of the gels with protein, some of these components can be seen to cross-react with the antiserum against chromogranin A in an immune replica and are therefore structurally related to it; possibly they could arise through modification of the species of M_r 70000 which is produced by in vitro translation. It could be argued that this is itself the result of proteolysis, but we have detected no components of higher or lower M_r, suggesting that no proteolysis of translation products has occurred in vitro. While this work was in progress a report appeared in which a chromogranin precursor of M_r 100000 was identified after translation of adrenal mRNA in a cell-free system derived from wheat germ [18]. It is possible that this species is also produced by translation in the reticulocyte system, but is for some reason not precipitated by the antiserum. However we have shown (fig.1D) that our antiserum reacts with some components of apparent

M_r greater than 70000; the small amounts of these in the immune precipitate (fig.2B) may simply reflect their scarcity in the granule lysate. If they were produced by in vitro translation, particularly if they were the sole product, they would presumably be quantitatively precipitated. An alternative explanation is that they are translated from different mRNA species, which are either lost during isolation, or not efficiently translated in vitro. They might even arise through extensive post-translational modification of the major translated species, of apparent M_r 70000.

We therefore conclude that the acidic proteins of M_r between 40000 and 65000 (the chromogranins) that are released from the matrix of chromaffin granules are derived by proteolysis of one or two high-M_r precursors. We have isolated the primary translation product of the messenger RNA for chromogranin A, a polypeptide of M_r 70000: its structural relationship to mature chromogranin A is now under investigation.

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